Biological Control of Gray Leaf Spot (*Pyricularia grisea* (Cooke) Sacc.) of Ryegrass

By

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BSc (Hons) University of KwaZulu-Natal

Submitted in fulfilment of the

Requirement for the degree of

Master of Science

In the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Sciences

College of Agriculture, Engineering and Sciences

University of KwaZulu-Natal

Pietermaritzburg

Republic of South Africa

February

2017

DISSERTATION SUMMARY

Gray leaf spot (GLS) is a common fungal disease of ryegrass, caused by *Pyricularia grisea* (Cooke) Sacc. The disease is especially devastating on newly planted ryegrass. GLS lesions develop profusely on leaves, leaf sheaths and stolons of ryegrass. When weather conditions are favourable for disease development, this disease is able to cause serious damage to already established grass. Cultural management practices are used to control GLS; however, they are often ineffective. Fungicides are considered to be the best available method for managing GLS but they have resulted in undesirable consequences such as the development of fungicide resistant *P. grisea* strains. Therefore, alternative control strategies and integrated disease management practices are needed to control disease spread on ryegrass.

A total of 87 bacterial isolates were obtained from various graminaceous species and screened *in vitro* for their activity against *P. grisea*. Two commercially available *Trichoderma* strains were also tested for their ability to inhibit *P. grisea* mycelial growth *in vitro*. During the secondary *in vitro* screening, 9 bacterial strains inhibited mycelial growth of *P. grisea* on PDA plates by 65.3–93.1%. The commercial *Trichoderma harzianum* strains, T.kd and T.77, inhibited mycelial growth *of P. grisea* by 80.2% and 82.8%, respectively. The nine bacterial isolates were sequenced and subjected to Blast analysis. Four of the nine isolates were identified as *Bacillus* species, two as *Pseudomonas* species, two as *Bacillus amyloliquefaciens* and one as *Bacillus cereus*.

The nine bacterial strains and the two commercial *T. harzianum* strains were further tested against GLS under greenhouse conditions. Azoxystrobin was used as a standard fungicide control. In experiments conducted on annual ryegrass the bacterial isolates were inconsistent in their performance. In Experiment 1, *Bacillus* spp S6 and B57 reduced GLS severity by 26 and 34%, respectively, while in Experiment 2, *Bacillus* spp M1 reduced disease severity by 20%; and *B. amyloliquefaciens* B7 and *Pseudomonas* spp I74 reduced it by 19%. However, the levels of reduction in disease severity were not significant in either experiments when compared to the pathogen inoculated control. Application of *T. harzianum* strains T.kd and T.77 did not significantly reduce GLS in

both experiments. Azoxystrobin reduced GLS severity by 28% in Experiment 1, with a slight increase to 32% in Experiment 2. In experiments on perennial ryegrass two isolates consistently reduced GLS severity when compared to pathogen inoculated control. *B. cereus* I48 and *Bacillus* spp S6 reduced GLS severity by 35% and 30%, respectively in Experiment 1 and by 41% and 39%, respectively in Experiment 2. T. *harzianum* strains T.kd and T.77 also did not significantly reduce GLS severity in perennial ryegrass. Azoxystrobin significantly reduced GLS severity by 35 % in Experiment 1 and by 54% in Experiment 2 ($P = 0.003$).

Four different concentrations of liquid potassium silicate (KSil) applied once or twice a week were evaluated for their effects on GLS under greenhouse conditions. The optimum concentration and application frequency was determined in an effort to use them in combination with biological control agents to manage GLS in the field. In the experiments conducted on perennial ryegrass, 300 ppm KSil applied once a week significantly reduced GLS severity by 23.5%. A 200 ppm concentration applied once a week significantly reduced GLS severity on perennial ryegrass by 29.8%.

From the greenhouse studies the best biocontrol agent and KSil treatment were selected for field evaluation. *Trichoderma harzianum* strain T.77 and azoxystrobin were also tested. Potassium silicate and the biocontrol agents were tested alone and as combined treatments. None of the KSil + bacterial isolates combinations effectively controlled GLS severity in the field. Potassium silicate and *T. harzianum* strain T.77 applied alone were also ineffective in reducing GLS severity in the field. *Bacillus* spp M1 reduced GLS severity (22%) on annual ryegrass, although not significantly. On perennial ryegrass *Bacillus cereus* I48 significantly reduced GLS severity by 36.6%. Azoxystrobin effectively controlled GLS on both grasses, significantly reducing disease severity by 44.0 and 53.6% on perennial and annual ryegrass, respectively ($P = 0.002$).

The *Bacillus* strains that were selected as the best biological control agents against GLS on ryegrass in the greenhouse showed potential in reducing GLS severity in the field when applied alone. Potassium silicate showed no ability to reduce GLS severity in

the field. The combination treatments were not effective in controlling GLS in the field. More field trials need to be conducted to ascertain the ability of the bacterial antagonists to reduce GLS disease severity on ryegrass.

DECLARATION

I, Nompumelelelo Dammie, declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research

2. This dissertation has not been submitted for any degree examination at any other university

3. This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted. Then:

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Signed: ……..
Miss N. Dammie

Signed: …….
Dr K.S. Yobo (Supervisor)

Signed: ……..
Prof. M.D. Laing (Co-supervisor)

ACKNOWLDEGEMENTS

I would like to express my sincere gratitude and appreciation to the following people and institutions:

My supervisor, Dr Kwasi S. Yobo, and co-supervisor, Professor Mark D. Laing, for their assistance and expert guidance throughout this study.

Dr Mike Morris for his constructive ideas and suggestions.

Mrs. Susan Van der Merwe for her advice and assistance with the greenhouse and field trials.

Mr Ian Doidge for his technical assistance with field trials.

Mr Richard Burgdorf for his help with the laboratory work.

Mr Matthew Erasmus for technical assistance with the greenhouse.

Mrs J. Khumalo, Mr J. Madondo, Mrs M. Khambula, Mr S. Mlotshwa, Mr T. Dlamini and Mr V. Dlamini for all their help and technical support in the greenhouse.

My fellow postgraduate colleagues in Plant Pathology for their support, advice and happy times in the laboratory and office.

The National Research Foundation for their financial support.

Barenbrug seed (Pty) Ltd and Farmers Agri-Care (Pty) Ltd for donating seed for the trials.

All my close friends for their positive influence, encouragement and prayers.

My parents and siblings for their prayers, support and encouragement.

Finally, my Lord and Saviour Jesus Christ, for His love and grace in my life.

DEDICATION

To my parents, Jethro and Busisiwe Dammie,
my brother, Benjamin Dammie,
and my sister Karabo Dammie.

Thank you for your love, encouragement and support throughout my study.

DISSERTATION INTRODUCTION

Annual ryegrass (*Lolium multiflorum* L.) and perennial ryegrass (*Lolium perenne* L.) are the commercially most important ryegrasses grown in cool temperate climates throughout the world (Spangenberg *et al*., 1998). They are widely distributed in Europe, North and South America, North, East and South Africa, Asia, Australia and New Zealand, where they are used for agricultural and recreational purposes (Spangenberg *et al*., 1998). They are important for grazing, stabilising soil and for multiple uses as forage, conservation and turf (Heijden and Roulund, 2010; Brazauskas *et al.*, 2013). A number of fungal pathogens that cause severe diseases of these grasses have emerged during the past two decades (Rahman *et al*., 2014). Among the most devastating diseases is gray leaf spot (GLS) caused by *Pyricularia grisea* (Cooke) Sacc. (Ma, 2006; Lemus and Tomaso-Peterson, 2010) (Teleomorph *Magnaporthe grisea*. (Hebert) Barr).

Newly planted grass are highly susceptible to the disease. Severe outbreaks of the disease occur during moderately warm temperatures of 26-29°C in periods of extended leaf wetness and relatively high humidity of >80% (Uddin and Vili, 2003). The use of cultural practices to control the disease are inadequate under high disease pressure. Although the use of fungicides is an effective method of control, the emergence of resistance strains pose a problem (Shi and Wang, 2008). Furthermore, there are no registered fungicides in forage production, thus creating limited disease management options for livestock farmers (Lemus and Tomaso-Peterson, 2010). The use of biological control agents and potassium silicate may provide an alternative control strategy for the management of GLS of ryegrass.

The aim of this research was to investigate the application of potential biological control agents and potassium silicate (KSil) applied singly or in combination for their ability to manage GLS under greenhouse and field conditions.

The specific objectives of this study include:

1. A literature review on GLS; the causal microorganism, its life cycle; epidemiology, symptoms, economic importance and control strategies;

2. To isolate and screen bacterial microorganisms *in vitro* for antagonism against GLS (*Pyricularia grisea*) of annual ryegrass and perennial ryegrass;

3. To evaluate the potential of the best antagonists in controlling GLS under greenhouse conditions;

4. To evaluate four different concentrations and two application frequencies of KSil to suppress GLS disease under greenhouse conditions;

5. To evaluate the efficacy of the best bacterial strains and best KSil concentration and frequency selected during greenhouse studies for the control of GLS under field conditions.

This dissertation is structured in the form of five chapters. Each chapter covers specific objectives of the research that was conducted. With the exception of Chapter One, the literature review, the other four chapters were independent studies and were written in the form of discrete research chapters, each following a stand-alone research paper. This format is the standard dissertation model that has been adopted by the University of KwaZulu-Natal because it facilitates the publishing of research out of the dissertation far more readily than the older monograph form of dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information between chapters.

This research was undertaken in the Discipline of Plant Pathology, at the University of KwaZulu-Natal, Pietermaritzburg Campus under the supervision of Dr K.S. Yobo and Prof M.D. Laing.

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CHAPTER ONE

LITERATURE REVIEW

1.1. Introduction

The genus *Lolium* is one of the most important groupings of grasses grown in the temperate climatic zones in the world (Charmet *et al.*, 1996). This group includes the widely cultivated annual ryegrass (*Lolium multiflorum* L.) and perennial ryegrass (*Lolium perenne* L.). Both perennial and annual ryegrasses are used for feeding ruminant livestock, whereas perennial ryegrass can also be used as an amenity grass in sport courses and residential areas (Brazauskas *et al.*, 2013).

Ryegrass blast, also called gray leaf spot (GLS), is caused by *Pyricularia* grisea (Cooke) Sacc. and is a destructive disease of ryegrass (Bangya, 2006; Lemus and Tomaso-Peterson, 2010). The main factors that favour *P. grisea* infection are: high temperatures, relatively high humidity and the presence of dew. GLS develops rapidly under these ideal conditions and as a result, entire ryegrass swards can be killed within a few days (Uddin and Vili, 2003).

P. grisea has a broad host range and infects more than 50 monocotyledonous species, including crops of economic importance such as wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and finger millet (*Eleusine coracana* (L.) Gaertn.) (Latha *et al.*, 2005; Peyyala and Farman, 2006). Additionally, *P. grisea* causes rice blast disease, which is one of the most severe and devastating diseases of rice (*Oryza sativa* L.) (Zhu *et al.*, 1999).

P. grisea produces specialized infection structures called appressoria to penetrate the leaves and stems of host plants. This allows the fungus to gain entry into the underlying tissue (Viaud *et al.*, 2002). Once established in the plant, the fungus switches to necrotic growth, killing the plant cells and branching out into the tissue (De Vleesschauwer *et al.*, 2006). Developing novel mechanisms to control GLS will require detailed understanding of both the disease and the biology of *P. grisea* (Viaud *et al.*, 2002).

The use of resistant cultivars and the application of fungicides have been employed to control GLS (Naureen *et al.*, 2009). However, the effectiveness of genetic resistance and chemical control alone are limited because of the frequent emergence of *P. grisea* mutants that are able to overcome specific resistance genes or fungicides (Shi and Wang, 2008). Moreover, the use of fungicides is expensive as well as environmentally undesirable (Naureen *et al.*, 2009). Therefore, it is important to search for alternative strategies to minimise GLS disease on ryegrass.

The effects of silicon (Si) in reducing the incidence of fungal diseases in plants has been known for some time (Fauteux *et al.*, 2005). Silicon has been demonstrated to enhance the tolerance of plants to biotic and abiotic stress in several graminaceous plants including rice and ryegrass (Romero-Aranda *et al.*, 2006; Nanayakkara *et al.*, 2008). It is also an environmentally safe disease control method (Alvarez and Datnoff, 2001).

Biological control of fungal pathogens with microorganisms has been investigated for more than 70 years and is becoming a realistic alternative to chemical treatment (Massart and Jijakli, 2007). It assumes special significance in being an eco-friendly and cost effective strategy for disease management that can also be integrated with other strategies, such as the development of host resistance to attain higher levels of protection against GLS (Karthikeyan and Gnanamanickam, 2008). Since biological control is a key component of integrated disease management the understanding of how biological control agents exert their protective effects is a prerequisite for their suitable selection, production, formulation and use (Massart and Jijakli, 2007).

1.2. Commercial use and economic importance of ryegrass

Annual and perennial ryegrass are native to Europe. The grasses are now used for forage and turf purposes throughout the temperate regions of the world including North and South America, South Africa, Australia and New Zealand (Yamada, 2013).

Perennial ryegrass, along with annual ryegrass, is extensively cultivated for feeding ruminant livestock for dairy, meat and wool production. Eighty percent (80%) of the world's bovine milk and 70% of the world's beef and veal are produced from temperate

grassland systems across the world (Wilkins and Humphreys, 2003). This grass has a superior herbage digestibility and grazing tolerance (Shinozuka *et al.*, 2011). Annual ryegrass is often included in permanent pasture mixtures to provide feed while the slower growing perennial ryegrass becomes established. Annual ryegrass also provides good winter growth, whereas perennial ryegrass has little to no growth in winter (Anonymous , 2008).

Perennial ryegrass is also widely used in amenity grasslands, including sports turf, golf courses, home lawns, parks and general landscape areas. This is due to its fast establishment, fine texture and dark green colour (Heijden and Roulund, 2010).
In the UK, the value of forage grass is measured by its end products, milk and meat, which amount to £6 billion per annum (King *et al.*, 2008). There is an increasing demand for meat and milk products as well as an increase in the application of ryegrass in leisure activities. Thus the economic value of this grass species is likely to increase (King *et al.*, 2008; Studer *et al.*, 2010). Below is a table showing the market value of both annual and perennial ryegrass seed in South Africa (Table 1.1).

Table 1.1: South African market value of annual and perennial ryegrass based on the retail selling price of seed (R millions) for the period 2012-2015.

Crop	**Year**		
	2012/13	**2013/14**	**2014/15**
Annual ryegrass[#]	43.17	41.06	55.96
Perennial ryegrass	31.70	31.03	43.32

Table amended from SANSOR market data 2013-2015 (SANSOR, 2012-2015).

exclude seed sales of turf grasses. No GMO's marketed within this division.

1.3. The Pathogen and Disease

1.3.1. Taxonomy and Morphology of *Pyricularia grisea*

Pyricularia grisea is classified in the Phylum Deuteromycota (Class Sordariomycete, Order Moniliales, Family Moniliaceae). It is the anamorphic stage of the fungus and is commonly found in nature. The sexual (teleomorph) stage of the fungus, *Magnaporthe*

grisea (Herbert) Barr, belongs to the Phylum Ascomycota, Order Diaporthales and has only been observed *in vitro (*Karthikeyan and Gnanamanickam*, 2008)*.

The anamorph, *P. grisea,* produces 3-celled, pyriform (pear-shaped) conidia arranged in clusters of three to five at the tip of the conidiophore (Lau and Hamer, 1998). The conidia are two-septate and hyaline to pale olive in colour. The conidiophores are single or in fascicles, simple, rarely branched, showing sympodial growth (Lau and Hamer, 1998). The teleomorph *M. grisea* was not known for a long time (Scheuermann *et al*, 2012). However it has been produced in the laboratory after crossing compatible isolates. The teleomorph stage produces hyaline ascospores, fusiform shaped with 3-septa and enveloped by a unitunicate ascus (Scheuermann *et al*, 2012).

1.3.2. Symptoms of the Disease

Gray leaf spot is most damaging on seedlings and young ryegrass. Disease symptoms first appear as small water-soaked lesions on the leaves that further develop into gray, grayish-brown, or light brown necrotic spots, with a purple to dark-brown border (Fig 1.1a) (Trevanthan *et al.*, 1994). Older lesions are often surrounded by a yellow chlorotic halo and are most common along the margin of the leaf blade (Figure 1.1b; Uddin and Vili, 2003). Foliar blighting occurs when multiple leaf spots coalesce and become irregular in shape (Uddin *et al.*, 2003). Dying leaves develop a characteristic twist at the leaf tip, giving a “fishhook” appearance (Fig 1.1c; Williams *et al.*, 2001). In the field, severely diseased plants appear as patches of dead ryegrass (Fig 1.1d; Trevanthan *et al.*, 1994).

Figure 1.1 Brown necrotic spots on ryegrass blade (a). Gray leaf lesions on ryegrass blade (b). Characteristic GLS twist at the tip of a dying ryegrass, giving a “fishhook” appearance (c). Patches of dead ryegrass (d). (Harmon and Latin, 2003).

1.3.3. Disease Epidemiology

The pathogen overwinters as conidia or dormant mycelia in older infected leaves, plant debris, either in or on seed (Uddin *et al.*, 2003). It is thought that these serve as the primary inoculum for infection in the early growing season. The conidia are spread by wind, dew droplets and equipment to infect new leaves. The disease is polycyclic and is highly dependent on favourable conditions for the fungus to infect new leaves (Fig1.2; Howard and Valent, 1996).

Gray leaf spot is favoured by warm, wet and humid conditions during late summer or in late spring (Uddin *et al.*, 2004). Severe outbreaks of the disease occur during periods of moderately warm temperatures of 26ºC - 29ºC (Douhan *et al.*, 2011). In addition, extended periods of leaf wetness and high relative humidity of >80% are essential for infection because it allows for maximum germination and infection by conidia. Under such favourable conditions GLS develops rapidly and entire ryegrass fields can be killed within a few days (Uddin *et al.*, 2004).

1.3.4. Infection process of *Pyricularia grisea*

P. grisea has been used as a model organism for the investigation of plant diseases caused by fungi, largely because of its economic importance (Wilson and Talbot, 2009). Foliar infection is initiated when the conidia of *P. grisea* attaches itself tightly to the host plant leaf cuticle by means of an adhesive that is secreted from an apical compartment of the conidium during hydration (Fig 1.3A; Wilson and Talbot, 2009). Once attached**,** the conidium germinates by rapid growth of a germ tube (Fig 1.3B). The tip of the elongated germ tube enlarges and forms a dome-shaped, melanin-pigmented infection structure called the appressorium that is essential for pathogenicity (Fig1.3C and D; Gupta and Chattoo, 2007). The appressorium then generates enormous turgor pressure focused on a sharp penetration peg that pierces through the cuticle layer (Fig 1.3E; Park *et al.*, 2009). Once the fungus has penetrated into the epidermal cells, it differentiates into specialised multicellular bulbous primary infectious hyphae (Fig 1.3F; Ribot *et al.*, 2008). After filling the initial epidermal cells, longer, more conventionally cylindrical hyphae branch out into adjacent tissue and the leaf is rapidly colonised (Fig 1.3G; Berruyer *et al.*, 2006).

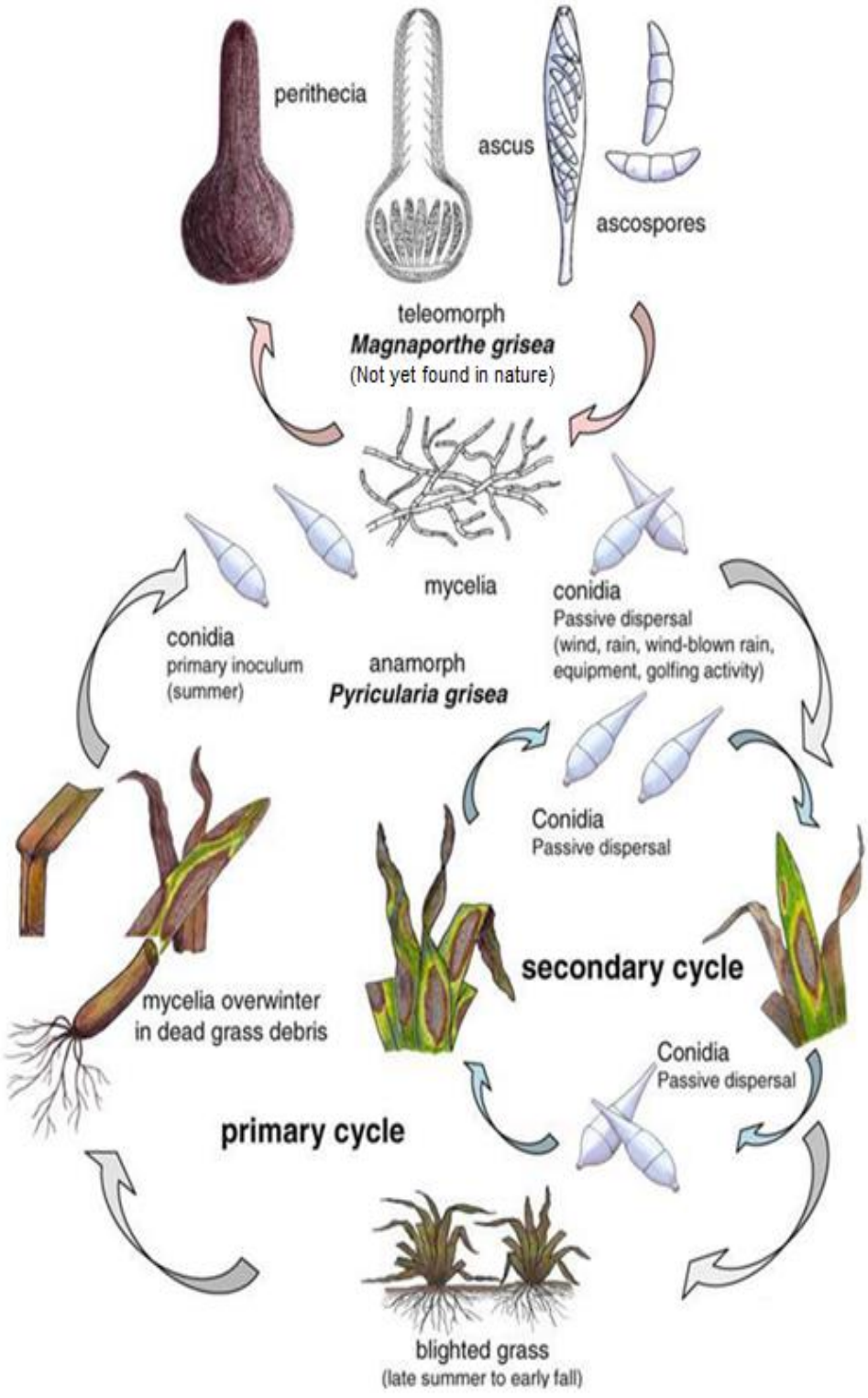

Figure 1.2 Life cycle of *Pyricularia grisea* (Uddin and Vili, 2003)

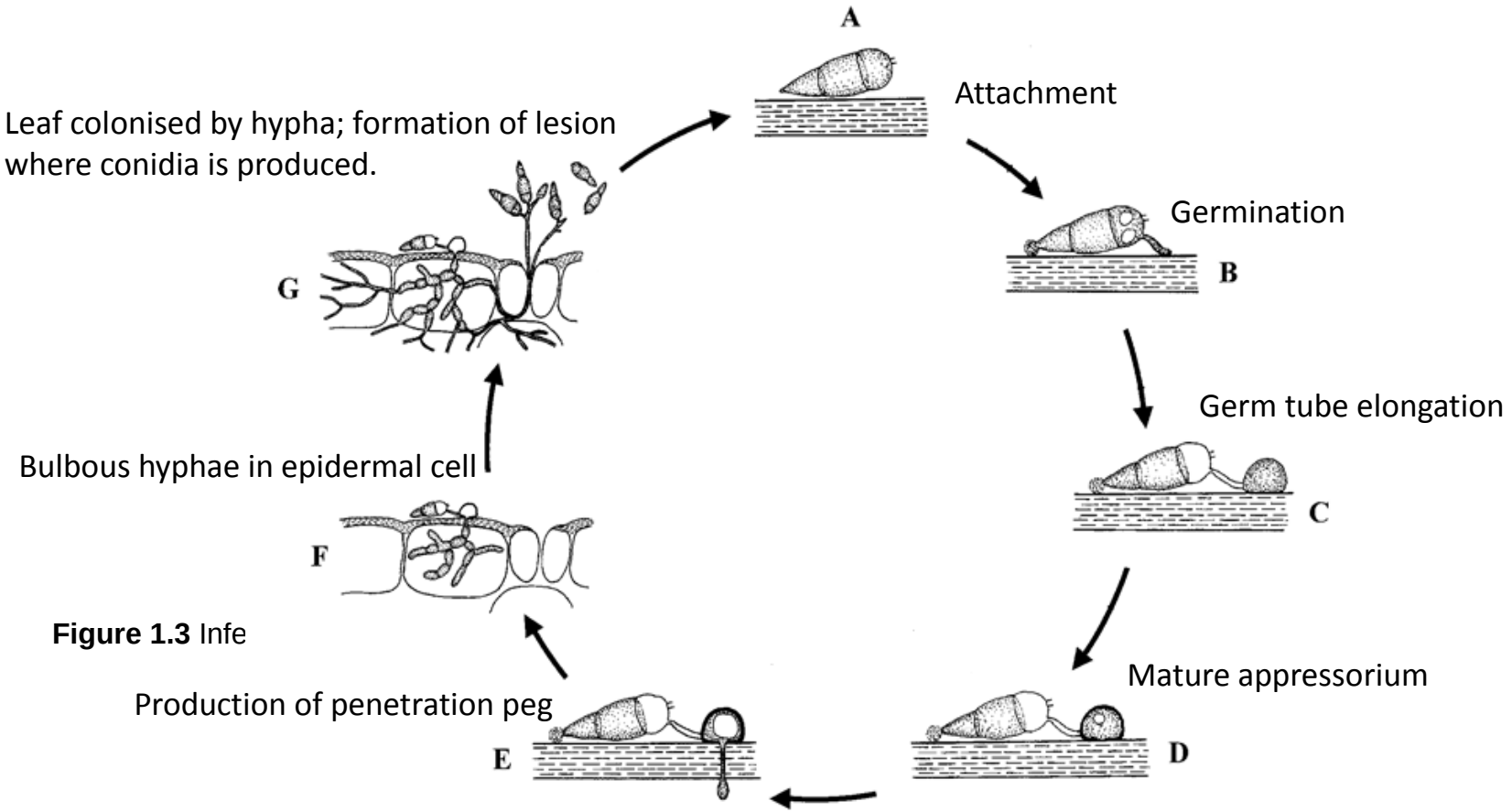

Figure 1.3 Infe

1.4. Disease Management

1.4.1. Cultural practices

Certain cultural control methods are known to influence the development of GLS. Cultural methods such as proper mowing, minimizing nitrogen fertilization and irrigation reduce the susceptibility of ryegrass to GLS (Williams *et al.*, 2001a). Lower mowing heights place additional stress on the ryegrass during summer, resulting in increased disease severity. Additionally, mowing height may have an impact on the microenvironment of the ryegrass canopy because taller canopies maintain higher levels of humidity within the lowest canopy regions for longer periods of time, thus creating a more suitable environment for pathogen activity (Williams *et al.*, 2001a). Since temperature and leaf wetness duration significantly affects disease severity and disease incidence, watering deeply and less frequently under warmer temperatures is beneficial in suppressing the pathogen (Williams *et al.*, 2001a). Unfortunately, cultural practices alone usually do not provide satisfactory control of GLS in ryegrass due to the rapid development of the disease and the high susceptibility of currently available ryegrass cultivars.

1.4.2. Control using fungicides

Control of GLS is dependent on the use of preventative fungicide treatment, even though only a few are effective in controlling the disease (Hoffman and Hamblin, 2000). Preventative fungicides used against GLS belong mainly to three fungicide classes: benzimidazole, demethylation inhibiting (DMI) and quinone outside inhibitor (Q_oI) classes. Fungicides such as thiophanate-methyl belong to the benzimidazole class while propiconazole, myclobutanil and triadimefon belong to the DMI class, and azoxystrobin, trifloxystrobin and pyraclostrobin belong the Q_oI class. The Q_oI fungicides are the most widely used preventative fungicides for the control of GLS disease (Kim *et al*., 2003; Brent and Hollomon, 2007).

The Q_oI fungicides bind to the quinol-oxidising (Q_o) site on cytochrome b, which forms part of the cytochrome b_1 complex, located in the inner mitochondrial membrane of fungi and other eukaryotes (Kim *et al.*, 2003). This action by the fungicide disrupts the

energy cycle within the fungus by halting the production of ATP, thus inhibiting respiration (Bartlett *et al.*, 2002).

A potential problem with the strong reliance on fungicide application is the development of fungicide-resistant strains (Park *et al.*, 2005). Q_oI fungicides have a highly specific mode of action. This increases the potential for selection of pathogens with a reduced sensitivity to these fungicides. Consequently, the widespread use of these fungicides has already resulted in the appearance of resistant strains (Kim *et al.*, 2003).

A further problem is that there are no labeled fungicides for use in forage production, thus livestock farmers have limited control options for managing the disease (Lemus and Tomaso-Peterson, 2010).

1.4.3. Resistant varieties

Host resistance is an effective and environmentally sound control strategy and has been extensively studied and used in other hosts of *P. grisea* (Mizobuchi et al., 2003). For example, in rice, the use of resistant varieties is one of the most important ways to control the rice blast disease (Mizobuchi *et al.*, 2003). Genetic resistance to blast is controlled by both major genes, and additive genes. More than 80 major blast resistance (R) genes have been documented, of which more than 19 have been cloned and characterized (Gupta *et al.*, 2012). Qualitative resistance is controlled by a single gene in both the host and the pathogen; however, this type of resistance is unstable and quickly breaks down. Thirty-five QTLs that control minor genes or partial resistance have been identified and documented (Jia and Liu, 2011). Quantitative resistance is more durable because it confers broad spectrum resistance against diverse races of the pathogen (Han *et al.*, 2006).

There have been improvements in the development of disease resistance in turfgrass genotypes. Quantitative resistance to GLS within perennial ryegrass has been identified in European and American germplasm (Jo *et al.*, 2007). Four QTLs for resistance to GLS have been identified while qualitative resistance appears to be absent in the current perennial ryegrass germplasm (Jo *et al.*, 2008). Lack of complete resistance in

ryegrass suggests that the receptor gene that allows them to recognize a particular *M. grisea* population that causes GLS is absent (Chauhan *et al.*, 2002).

Another approach to control of GLS through host resistance would be to identify receptor genes in other monocot species and introduce them to perennial ryegrass (Chauhan *et al.*, 2002). The Pi-CO39(t) locus in one variety of rice (CO39) confers resistance to *P. grisea* isolates carrying the AVR1-CO39(t) avirulence locus (Peyyala and Farman, 2006). This locus that confers avirulence is conserved in non-rice (cereals and rice) infecting isolates of *P. grisea* (Chauhan et al., 2002). *P. grisea* isolates that infect perennial ryegrass are unable to infect this variety of rice, suggesting that the pathogen possess genes that elicit a defense response in the host (Peyyala and Farman, 2006). Therefore, it is suspected that the perennial ryegrass isolates may contain the corresponding AVR1-CO39 gene and if it does, then Pi-CO39(t) would be useful for engineering *P. grisea* resistance in perennial ryegrass (Peyyala and Farman, 2006).

1.5. Biological control

Beyond good agronomic and horticultural practices, the prevention and control of plant diseases has over the years become highly dependent upon the use of agrochemicals (Pal and Gardener, 2006). This is due to their effectiveness and ease of utilization (Kumar *et al.*, 2011). However, the excessive use and misuse of agrochemicals has caused many problems. Pollution of soils and ground water reservoirs, accumulation of undesirable chemical residues in the food chain, emergence of fungicide resistant pathogen strains, and negative effects on the health of the growers are all potential problems (Jamalizadeh *et al.*, 2011). Consequently, there is a strong demand from consumers and authorities for safer, and more rational, sustainable and eco-friendly strategies (Brimmer and Boland, 2003). Biological control through application of bioactive agents or microorganisms antagonistic to plant pathogens offers an eco-friendly, cost effective and sustainable approach for disease management. Additionally, when used in conjunction with other strategies such as host resistance, it affords an even greater level of protection against plant diseases (Karthikeyan and Gnanamanickam, 2008). Even though the performance of many biological control

agents may not be to equal that of an excellent fungicide, some biological control agents have been reported to be as effective as fungicides (Elad *et al.*, 1996). There is little information concerning the use of biological agents for the control of GLS of ryegrass. However, biological control of rice blast has been attempted with various microorganisms including: Actinomycetes, *Pseudomonas* and *Bacillus* species (Table 1.2) (Shan *et al.*, 2013).

1.5.1. Mechanisms of biological control agents

The various mechanisms of action employed by antagonistic bacteria and fungi on plant pathogens have been the subject of many studies in the field of biological control (Janisiewicz *et al.*, 2000). A mechanism of action can be described as the strategy used by a beneficial microorganism against a disease-causing pathogen (Liu *et al.*, 2010). A successful biological control agent will employ several mechanisms of action in controlling disease development (Jamalizadeh *et al.*, 2011). Mechanisms of biological control include: antibiosis, mycoparasitism, competition for space and limited resources and induced systemic resistance of the host plant (Zhu *et al.*, 2011).

1.5.2. *Bacillus* species as biological control agents

Several strains of the genus *Bacillus* have received much attention as biological control agents because of their advantages over other gram-negative bacteria, and fungal biological control agents (Bargabus *et al.*, 2004). Many *Bacillus* species have shown to be capable of producing several broad-spectrum antimicrobial compounds, including: peptide antibiotics, hydrolytic enzymes and β-glucanases, which are potent enzymes for degrading fungal cell walls (Leelasuphakul *et al.*, 2006). Lipopeptides produced by *Bacillus* are also known to play a role in reinforcing host resistance (Shu-Bin *et al.*, 2012). *Bacillus* species form dormant endospores that are tolerant to heat and desiccation. This unique property of *Bacillus* species allows for the easy development of commercial formulations of the bacteria (Lee *et al.*, 2006). Furthermore, their lack of pathogenicity to plants makes this group of bacteria attractive as potential biological control agent (Shan *et al.*, 2013).

1.5.3. Fluorescent *Pseudomonas* as biological control agents

Fluorescent *Pseudomonas* strains are considered as one of the most important and effective genera of biological control agents (Abo-Elyousr and El-Hendawy, 2008). They have been used primarily for seed, soil and foliar treatment due to their efficient antagonistic activity against various plant pathogens (Anand *et al.*, 2010). This group of bacteria control plant pathogens by several mechanisms of action such as antibiotic production, production of secondary metabolites, production of siderophores and lytic enzymes (Anand *et al.*, 2010). Furthermore, fluorescent *Pseudomonas* strains are presumed to induce systemic resistance, as well as to promote the growth and development of plants (Nandakumar *et al.*, 2001).

1.5.4. *Trichoderma* species as biological control agents

One of the most widely used microorganisms of fungal biocontrol is the genus *Trichoderma.* Several species are important: *Trichoderma harzianum Rifai*, *T. virens* Arx and *T. viride* Pers (Díaz *et al.*, 2012). They possess a high reproductive capacity and the ability to survive under a wide range of environmental condition (Woo *et al.*, 2005). *Trichoderma* species are able to colonise soils, and the rhizosphere and phyllosphere of many plants (Longa *et al.*, 2008). They have been successfully used for biocontrol of pathogens on plant surfaces of cruciferous, solanaceous and graminaceous plants (Perello *et al.*, 2003). A number of commercial formulations using a variety of *Trichoderma* strains are available for crop production worldwide (Table 1.3).

The mechanisms by which *Trichoderma* acts as a biological control agent are many and complex. These mechanisms include direct competition for space and nutrients, mycoparasitism mediated by secretion of enzymes that degrade the pathogen's cell walls, and the production of antibiotics and other secondary metabolites that inhibit pathogen growth. Moreover, *Trichoderma* species have a strong ability to promote plant growth and defense mechanisms in plants (Samuels, 2006).

Table 1.2 Examples of *Bacillus* and *Pseudomonas* species used as antagonists against *Pyricularia grisea.*

Host	Antagonist	Mode of application	Comments	Reference
Rice	*P. chlororaphis* EA105	Root treatment	Isolate reduced the number of blast lesions by 33%. EA105 also triggers jasmonic acid and ethylene depended induced systemic resistance response in rice.	(Spence *et al.*, 2014)
Rice	*P. fluorescens Pf7-14*	Applied as seed treatment followed by 3 foliar applications	68.50% suppression of rice blast in seedbed experiment. 59.60% suppression of rice blast in field experiment.	(Krishnamurthy and Gnanamanickam, 1998)
Rice	*B. methylotrophicus* BC79 culture filtrate	Foliar application	Active substrate: phenaminomethylacetic acid. 89.87% suppression of rice blast in greenhouse experiment and 84.80% suppression of rice blast disease in field experiment.	(Shan *et al.*, 2013)
Foxtail millet	*B. polymyxa* KRU-22 and VLB-17 *P. fluorescens* Pf-52 and Pf-34	Foliar application	In the field strain Pf-52 and KRU-22 suppressed disease by 86.64% and 87.87% in *Setaria italica* line 1 and 86.64% and 83.21% respectively in line 2. Mixture of all 4 strains suppressed disease by 88.87% and 88.80% respectively in line 1 and line 2.	(Karthikeyan and Gnanamanickam, 2008)
Ryegrass	*P. aeruginosa*	Seed treatment and foliar application	Seed treatment: no significant suppression of the disease. Foliar spray: for all bacterial isolates disease incidence ranged from 22.60-48.40% which was significantly lower than control 75.20%. Three isolates, B12, B15, & B38 significantly suppressed GLS regardless of application intervals.	(Uddin, et al., 2003)
Rice	A2 *(B. firmus* E65) A6 *(B. firmus* E65, *B. cereus* II.14 *and P. aeruginosa* C32b*)*	Seedling root dip method and foliar application	A2 and A6 provided slower blast disease progression with the percentage of blast severity at 28.68% and 35.37% respectively. Disease severity for untreated control was 57.85%.	(Suryadi *et al.*, 2013)

Table1.3: Examples of commercial formulations using a variety of *Trichoderma* species

Biological control agent	**Trade name**	**Target disease/ organism**	**Crop**	**Company/ Reference**	**Country**
Trichoderma fertile Bissett	TrichoPlus®	*Rhizoctonia solani, Pythium* sp*, Sclerotinia* sp*, Fusarium* sp*, Phytophthora*	All crops	(BASF, 2014)	South Africa
T. harzianum Rifai kd	Eco-T®	Root diseases + enhances growth of plants	Vegetables, ornamentals and turf sp	(Plant Health Products, 2014)	South Africa
T. harzianum B77	Eco-77®	*Botrytis*, *Eutypa*	cucumber, tomato, grapevine	(Plant Health Products, 2014)	South Africa
T. viride Pers.	Gmax Tricon®	Soilborne plant pathogenic fungi	All crops	(Greenmax Agro Tech, 2014)	India
T. harzianum T-22 *and T. virens* G-44	Turfshield Plus®	*Pythium, Rhizoctonia, Fusarium* sp*, Sclerotinia homoeocarpa*	Turfgrass	(BioWorks, 2014)	USA
T. harzianum T-22	Rootshield®	*Pythium* sp*, Rhizoctonia, Fusarium, Thielaviopsis, Cylindrrocladium*	Turfgrass	(BioWorks, 2014)	USA
T. virens	Bioveer®	Root rot, shoot rot, damping off, wet rot	Pulses, oil seed, cucurbitaceous crops, solanaceous crops, cole crops and rice	(Ambika Biotech + Agro Services, 2014)	India
T. koningii Oudem. + *T. harzianum*	Promot®	*Rhizoctonia, Pythium sp, F. solani* + *F. oxysporum, Sclerotium rolfsii*	All crops	(SW- Dungesysteme GmbH, 2010)	Germany

1.6. The use of silicon in disease management

1.6.1. Silicon in Soil

Silicon (Si) is the second most prevalent element after oxygen in the earth's crust. However, certain soils tend to be low in plant-available Si (Nanayakkara *et al.*, 2008). Soils that are low in Si are typically highly weathered, leached, acidic and low in base saturation. Silicon deficiency occurs most often in Oxisols and Ultisols, which make up 34% of the area of major soils in Asia, Africa and America (Alvarez and Datnoff, 2001).

In ryegrass, repeated cropping and the removal of grass clippings may reduce the level of plant- available Si in the soil (Nanayakkara *et al.*, 2008). For plants such as rice where supplemental Si is required for maximum production, repeated cropping can reduce the levels of plant-available silicon.

1.6.2. Silicon uptake mechanisms

Silicon is readily taken up by plant roots as silicic acid ($Si(OH)_4$), an uncharged monomeric molecule at pH values below 9 (Schaller *et al.*, 2013). In leaf blades and leaf sheaths, ($Si(OH)_4$) mainly polymerises forming silica gel ($SiO_2.nH_2O$) (Schaller *et al.*, 2013). Plants differ in their ability to accumulate Si. Silicon concentrations vary in above ground plant parts, ranging from 0.1% to 10% w/w Si on a dry weight basis (Ma and Yamaji, 2006). This wide variation in Si concentration in plant tissue can be attributed to the ability of the roots to take up Si (Wu *et al.*, 2006). Three different Si uptake modes have been proposed: active, passive and rejective uptake (Mitani and Ma, 2005). Species that contain more than 1.0% w/w of Si show active uptake, and those with a rejective type have less than 0.5% w/w Si and tend to exclude it from their tissue. Plant species which contain between 0.5 and 1.0% w/w of Si are passive in their uptake of Si (Ma *et al.*, 2007b). Rice is a highly Si-accumulating species and therefore Si uptake is an active process, but some dicots such as cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), strawberry (*Fragaria ananassa* L.) and soybean (*Glycine max* L.) take up Si passively while tomato (*Solanum lycopersicum* L.) is believed to exclude Si. Other examples of graminaceous plants that take up Si actively besides rice include wheat, ryegrass and barley (Liang *et al.*, 2007). The variation in Si accumulation in the

different plant species has been attributed to the ability of the roots to take up Si (Mitani and Ma, 2005).

Two genes [*low silicon rice1* (*Lsi*1)] and *Ls*i2 encoding Si transporters have been identified in rice (Yamaji *et al.*, 2008). The *Lsi*1 gene encodes an aquaporin-like membrane protein, which is an influx transporter of silicic acid, while *Lsi2* encodes an active efflux transporter of silicic acid (Ma *et al.*, 2007a). Both genes are constitutively expressed in the roots and both transporters are localised in the root exodermis and endodermis with Lsi1 on the distal side and Lsi2 on the proximal side (Yamaji *et al.*, 2008). These proteins are responsible for transporting the Si in the soil solution into the xylem where a third transporter, Lsi6, transports silicic acid from the xylem into the xylem parenchyma cells (Yamaji *et al.*, 2008). Lsi6 is expressed in the leaf sheaths and leaf blades as well as the root tips and influences Si distribution to the rice shoots (Yamaji *et al.*, 2008).

1.6.3. Silicon in controlling fungal diseases

Reductions in the incidence and severity of soil-borne and foliar diseases due to the application of Si have been widely reported (Nanayakkara *et al.*, 2009). The favourable effects of Si in controlling fungal diseases of monocotyledons, mainly rice and other grasses, have been documented since the 1960s (Romero *et al.*, 2011). In rice, Si is deposited beneath the cuticle to form a cuticle-Si double layer in the leaves. This layer is believed to prevent physical penetration and makes the cell wall less vulnerable to enzymatic degradation by fungal pathogens (Datnoff *et al.*, 1997). Silicon accumulates around the infection peg at the point of pathogen penetration, thus inhibiting hyphal growth and haustoria formation (Datnoff *et al.*, 1997). Not only does Si create a mechanical barrier against pathogen entry, but Si is also able to prime host resistance (Fauteux *et al.*, 2005). The colonisation of rice by *P. grisea* has been limited as a result of an accumulation of glucanases, peroxidases, phenolics, lignin and PR-1 transcripts at the infection site (Ma and Yamaji, 2008). Table 1.3 shows examples of the enhancement of disease resistance in rice, perennial ryegrass, Bermuda grass (*Cynodon dactylon* L.) and St. Augustine grass (*Stenotaphrum secundatum* [Waltz] Kuntze) as a result of increased concentrations of Si in the tissues of these crops.

Table 1.4 The influence of silicon on diseases infecting various grass species

Grass species	Disease	Pathogen	Presence of silicon in leaf tissue	Effects of silicon application on disease levels	Reference
St. Augustine grass	GLS	*Pyricularia grisea*	For greenhouse studies: a soil concentration of 210mg.L^{-} rate of calcium silicate was needed to obtain 1.1% of leaf tissue silicon levels in susceptible cultivar.	In susceptible cultivar, the number of lesions reduced by 61% and the % of leaf area diseased reduced by 57%. In the resistant cultivar the number of lesions decreased from 43-57%.	(Brecht *et al.*, 2004)
Bermuda grass	Leaf spot	*Bipolaris cynodontis*	Percentage of Si in leaf tissue increased by 80% compared with the non-amended control.	Leaf spot severity was reduced by 38.9%	(Datnoff and Rutherford, 2003)
Perennial ryegrass	GLS	*Pyricularia grisea*	Soils with the highest calcium silicate rate showed tissue Si content of plants in Site 1 increases by 1% and those from Site 2 increased by 0.8%.	Final disease severity decreased significantly	(Nanayakkara *et al.*, 2009)
Rice	Blast	*Magnaporthe grisea*	In the wild-type leaves, Si accumulation was enhanced 3-fold compared to the wild-type which was in Si deficient soils	Blast lesion formation was inhibited in the wild-type (13.9 per plant) compared to the wild type (25.0 per plant) which was in Si deficient soils.	(Nakata, 2008)

1.6.4. Other benefits of Silicon

Silicon has been shown to enhance resistance in plants to abiotic stresses (salinity, metal toxicity, drought, radiation damage, nutrient imbalances, high temperature and freezing conditions) (Romero-Aranda *et al.,* 2006; Kim *et al.*, 2012). Stressful conditions negatively affect plant cells by causing the generation of reactive oxygen species (ROS) (Li *et al*., 2007; Lizan *et al*., 2009; Beckmann *et al*., 2012). Silicon is associated with increasing antioxidant defense abilities in plants induced by an over production of ROS (Shen *et al*., 2010). Silicon also accumulates in the cell walls of leaves, stems and roots, thereby reducing the intake and translocation of toxic ions from the roots to the shoots in soils with nutrient imbalances (Farooq *et al*., 2015).

The use of Si for disease management may reduce fungicide applications and therefore their environmental impact on agricultural land and water (Alvarez and Datnoff, 2001). In addition, Si sources have residual effects that persist over time. Therefore, this may reduce the need for subsequent applications significantly after the first treatment. Thus yearly application of Si may not be necessary, making application of Si economically feasible (Alvarez and Datnoff, 2001).

1.7. References

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CHAPTER TWO

Isolation and *in vitro* screening of potential biological control agents against gray leaf spot of ryegrass

Abstract

A total of 87 bacterial isolates were isolated from four graminaceous hosts and screened for their efficacy against *Pyricularia grisea*. During the *in vitro* primary screening test, only 18 isolates showed mycelial growth inhibition of *P. grisea,* with zones of inhibition ranging from 63.5 – 85.0%. Nine isolates were identified as the most promising antagonist against *P*. *grisea* based on their zones of inhibition in the secondary screening. The bacterial isolates showed different inhibition levels, ranging from 65.3 - 93.1% (P = 0.001). The nine isolates were amplified using 16S rRNA primers BacF and R1378 and the PCR products were sequenced. Four of the nine isolates were identified as *Bacillus* species, two *Pseudomonas* species, two as *Bacillus amyloliquefaciens* and one as *Bacillus cereus* when their sequences where subjected to Blast analysis. The results suggest that the isolated bacillus and pseudomonas spp have the potential to be used as biological control agents against *P.grisea* The potential of these isolates were tested on ryegrass under greenhouse conditions in Chapter Three.

2.1. Introduction

Gray leaf spot (GLS) of ryegrass [*Lolium L.* (*Poaceae*)] caused by the fungus *Pyricularia grisea* (Cooke) Sacc. is one of the most serious diseases of annual (*Lolium multiflorum* Lam.) and perennial (*Lolium perenne* Lam.) ryegrass (Bangya, 2006; Lemus and Tomaso-Peterson, 2010; Rahman *et al* 2015). Ryegrass seedlings are more susceptible to infection than established turf under unfavourable conditions. However, under favourable conditions the pathogen may cause extensive damage to mature ryegrass, resulting in low forage quality and serious yield losses. (Rahman *et al* 2014).

The management of the disease is achieved primarily through chemical and cultural control. However, under rapid disease development, cultural management practices

do not provide adequate control of gray leaf spot (Strobel, 2006). The development of resistance to fungicides among fungal pathogens limits the effectiveness of fungicides in controlling the disease (Vincelli and Dixon, 2002). The Qi fungicides are the most widely used preventative fungicides for the control of GLS and the widespread application and use of these chemicals has resulted in the appearance of resistant strains (Kim *et al*., 2003; Castroagudin *et al*., 2015).

Biocontrol with specific fungal and bacterial antagonists is a promising alternative to fungicide for the control of plant pathogens (Nega, 2014). This approach to disease control has been the focus of intensive research throughout the world (Singh *et al*., 2012). Various bacterial genera such as actinomycetes (Ningthaijam *et al*., 2009), *Pseudomonas* (Viji *et al*., 2003; Knaak *et al*., 2007) and *Bacillus* (Suryadi *et al*., 2013: Rahman *et al*., 2015), as well as species of the fungal genera *Trichoderma* (Singh *et al*., 2012) have been described as promising biological control agents antagonistic to *Pyricularia grisea*.

The objective of this study was to isolate and identify antagonistic bacteria and evaluate their efficacy in controlling *P. grisea in vitro*.

2.2. Materials and Methods

2.2.1. Isolation of *Pyricularia grisea* from infected ryegrass leaves

Perennial ryegrass samples with GLS lesions were collected from Cedara College of Agriculture (Hilton, KwaZulu-Natal, South Africa) and placed in brown paper bags and transported to the laboratory. Leaf sections with gray leaf spot lesions were surface sterilised with a 2% sodium hypochlorite solution for 2 min and washed in double sterilised distilled water for another 2 min. These were further treated with absolute ethanol for 2 min and washed again for 2 min in double sterilized distilled water. The leaf sections were then removed and placed on a sterile filter paper and left to air dry under a laminar flow. The dry leaf sections were plated onto oat meal agar (OMA) and incubated for 7-14 d at 28°C. Suspected *P*. *grisea* colonies developing from the samples were sub-cultured onto fresh OMA plates to obtain pure fungal cultures. Presumptive identification was done using a light microscope based on the shape, colour and size of the conidia. For long-term storage of the isolate, square pieces of filter paper were placed around actively growing edges of the fungal colonies on OMA plates. After the fungus had completely colonised the

pieces of filter paper, they were removed under aseptic conditions and placed in a 45 mm diameter Petri dishes and air-dried in a desiccator for 2 wk and stored at -20 °C until required.

2.2.2. Sample collection and isolation of biological control agents

Leaf samples were collected from four graminaceous plants, maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench.), sugarcane (*Saccharum officinarum* L.) and rice (*Oryza sativa* L.) collected from Ukulinga Research Farm (University of KwaZulu-Natal, Pietermaritzburg, South Africa) and placed in brown paper bags and transported to the laboratory. The leaf samples were cut into small pieces, rinsed using distilled water to remove soil particles and transferred into 20 ml McCartney bottles containing 9 ml of sterile distilled water. The McCartney bottles were heated in a water bath at 80°C for 30 min for isolation of *Bacillus* spp. Serial dilutions (10^1 - 10^4) from heated solutions were made after they had cooled. Aliquots of 0.1 ml were taken from each of the dilutions and plated on Nutrient agar (NA, Merck). For isolation of *Pseudomonas* spp, the leaf samples were cut into small pieces rinsed using distilled water to remove soil particles and transferred into 20 ml McCartney bottles containing 9 ml of sterile distilled water. The McCartney bottles were shaken at 150 rpm in an orbital shaker incubator for 30 min. Serial dilutions were prepared from the solutions and 0.1 ml of each dilution (10^1 – 10^4) was grown on Kings B Medium (20g L^{-1} peptone; 1.5g L^{-1} di-potassium phosphate; 1.5g L^{-1} magnesium sulfate; 15g L^{-1} glycerol; 15g L^{-1} agar; 100µg ml^{-1} chloramphenicol). The plates were incubated for 7 d at 28 °C. Morphologically different colonies were sub-cultured, purified and stored in 30% glycerol at -80 °C for subsequent use.

2.2.3. In vitro screening of bacterial isolates against *Pyricularia grisea*

The *in vitro* inhibition of mycelial growth of *P. grisea* by bacterial isolates was done using the dual culture technique described by Paulitz et al. (1992) and Idris et al. (2007). Three antibiotic paper discs were placed at equidistant points along the margins of potato dextrose agar (PDA) plates and a 0.1 ml suspension of the bacterial isolates was separately placed on each paper disc. The plates were then incubated for 48 h at 28 °C. A 4 x 4 mm^2 agar square carrying a mycelial plug of *P. grisea* was placed at the centre of the plate for each bacterial isolate and incubated at 28 °C for 7 d. There were 3 replicates. The radii of the fungal colonies towards

and away from the bacterial incorporated antibiotic paper disks were measured. Growth inhibition was calculated using the following formula:

% inhibition = (R-r)/ R x 100; where:

r = the radius of the fungal colony opposite the bacterial colony.

R = the maximum radius of the fungal colony away from the bacterial colony.

Isolates with more than 50% mycelia growth inhibition against the pathogen were considered as effective.

2.2.4. In vitro screening of *Trichoderma harzianum* kd and *Trichoderma harzianum* B77 against *Pyricularia grisea*

Two commercially available fungal biological control agent formulations viz., active ingredients *T. harzianum* Strain kd and *T. harzianum* Strain B77 were included in the screening of biological control agents. For the dual culture test, mycelial disks (4 x4 mm^2) were cut from actively growing colonies of the pathogen and the *T. harzianum* strains and were placed opposite each other in Petri dishes containing PDA. Petri dishes inoculated with *P. grisea* alone served as the control. Each treatment was replicated three times and incubated for 7 d at 28°C in the dark. The growth inhibition was calculated by using the formula:

$$PI = \frac{(C-T)}{C} \times 100$$

where

PI= Percent inhibition over control

C= diameter of *P. grisea* in the control

T= diameter of *P. grisea* in the dual plate

2.2.4. Biochemical and Molecular characterisation of bacterial isolates

Bacterial isolates were biochemically characterised using the Gram stain and the KOH test. For molecular characterization the 16S rRNA gene fragments from the different bacterial isolates were amplified according to the method of (Garbeva *et al*. 2003) using BacF, a *Bacillus* specific forward primer, in conjunction with R1378, a universal 16S rRNA reverse primer (Heuer *et al*., 1997) (Table 2.1). Each 25 µl reaction volume contained (1x) GoTaq® Flexi buffer, 1.75 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of primer, 1.25 U of GoTaq® DNA polymerase, approximately 50-100 ng template DNA made up to a final volume of 25 µl with nuclease-free water. Control reactions without DNA template were included in each round of amplifications. Thermal cycling was performed as follows: an initial denaturation,

94ºC for 5 min; followed by 30 cycles of denaturation (94ºC for 1 min), annealing (65ºC for 90 s) and extension (72ºC for 2 min); with a final extension of 72ºC for 10 min. All samples were kept at 4ºC. PCR amplification of the targeted gene fragment (~1300 bp) was confirmed by agarose gel electrophoresis. The resultant amplicons were sent for sequencing (Inqaba Biotech^{TM}, Hatfield Pretoria, RSA), where they were purified (Wizard PCR Prep Kits, Promega) before being sequenced using the ABI PRISM Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA). Both the forward and reverse primers were used and the amplified sequences were analysed with an ABI 3130*XL* sequence analyser (Applied Biosystems, Foster City, California, USA).

Table 2.1 Primers used in identifying the bacterial isolates selected from *in vitro* screening studies

16S rRNA primers	**Sequence (5´-3´)**	**Product size (bp)**	**Reference**
P1- BacF	GGGAAACCGGGGCTAATACCGGAT	24	Garbeva *et al*., (2003)
P2- R1378	CGGTGTGTACAAGGCCCGGGAACG	24	Heuer *et al*., (1997)

2.2.5. Statistical analysis

All data sets from the secondary screening results were subjected to analysis of variance (ANOVA) using GENSTAT (14th edition). Differences between treatment means were determined using Duncan's Multiple Range Test at a 5% significance level (P = 0.05)**.**

2.3. Results

2.3.1. Primary screening of potential bacterial control agents against *Pyricularia grisea*

A total of 87 bacterial isolates were used during the primary screening test. Only 18 isolates resulted in ≥ 50% growth inhibition of *P. grisea* (Table 2.2). The control plates without bacterial isolates were completely covered by the pathogen. The bacterial isolates that were antagonistic to *P. grisea* were further tested to confirm their biocontrol activity against *P. grisea.* The 18 isolates were selected for secondary screening based on the size of inhibition zones ≥ 50 percent.

2.3.2. *In vitro* secondary screening of selected biological control agents antagonistic to *Pyricularia grisea*

Eighteen isolates were selected based on their ability to inhibit *P. grisea* mycelial growth during the primary screening test. All the bacterial isolates and the *Trichoderma* strains significantly (P = 0.001) reduced the pathogen mycelial growth compared to the Control (Table 2.3). The bacterial isolates showed a percentage inhibition ranging from 54.05 – 93.1%. The highest level of inhibition was caused by Isolate B57. A change in the appearance of the mycelia (white to brown) opposite the bacterial isolates was observed in all the agar plates except for Isolate I48.

Table 2.2. *In vitro* primary screening of bacterial isolates as potential biological control agents against *P. grisea*.

Treatment	Isolate Type	% Inhibition
I48	Bacterium	63.5
I74	Bacterium	64.5
I101	Bacterium	68.23
I108	Bacterium	54.3
I120	Bacterium	78.8
I122	Bacterium	56.7
I124	Bacterium	69.4
I125	Bacterium	72.0
I126	Bacterium	66.7
I145	Bacterium	82.0
S2	Bacterium	56.0
S3	Bacterium	53.3
S6	Bacterium	74.7
M1	Bacterium	74.6
M7	Bacterium	73.7
B7	Bacterium	70.56
B8	Bacterium	83.6
B57	Bacterium	85.0
Other isolates	Bacterium	<50.0
Control	*P. grisea* only	0

Table 2.3: *In vitro* secondary screening of 18 bacterial isolates and two *T*. *harzianum* strains against *P. grisea*

Treatment	Isolate Type	% Inhibition* ±SE
S3	Bacterium	54.05±2.17b
I122	Bacterium	57.99±1.31bc
S2	Bacterium	58.73±2.68bc
I108	Bacterium	59.41±2.33bcd
I126	Bacterium	60.15±0.74bcd
I101	Bacterium	64.29±2.13cde
I48	Bacterium	65.3 ± 3.84de
I125	Bacterium	67.20±0.88ef
I124	Bacterium	72.2 ± 1.16fg
M7	Bacterium	73.28±1.09g
I74	Bacterium	75.2 ± 0.95gh
S6	Bacterium	80.9 ± 3.0hi
I145	Bacterium	81.54±2.54ij
B8	Bacterium	83.3 ± 2.67ij
M1	Bacterium	84.47 ± 2.63ij
B7	Bacterium	86.3 ± 2.13ij
I120	Bacterium	87.6 ± 1.53jk
B57	Bacterium	93.1 ± 1.73k
T. *harzianum*-77	Fungi	82.8 ± 0.88ij
T. *harzianum*-kd	Fungi	80.2 ± 0.92hi
Control	*P. grisea* only	0a
F value		96.66
P-value		0.001
SED		2.833
CV%		5.0%

*= Each value is reported as an average of three replicates; ± Standard error followed by the same letters are not significantly different based on Duncan's Multiple Range Test at 5% significance level (P = 0.05)

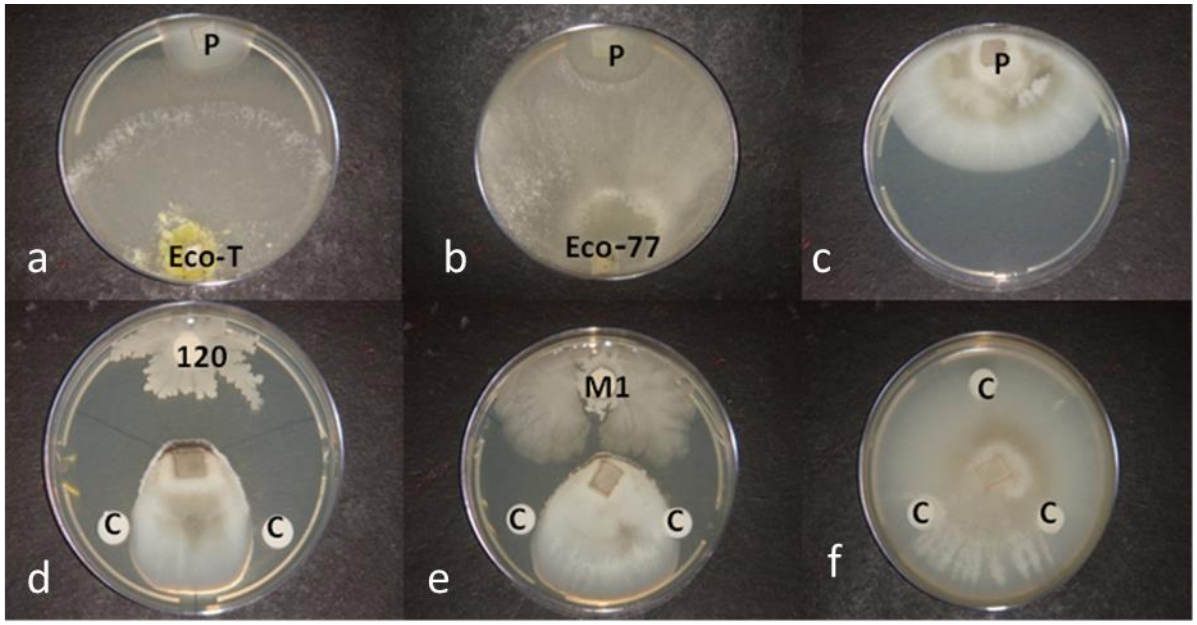

Figure 2.1. *In vitro* inhibition by two commercial *Trichoderma* strains, *T*. *harzianum* kd and *T*. *harzianum* -77 (a and b) and bacterial isolates, I120 and M1 (d and e) against *P. grise*a. Control plates with *P. grisea* only (c and f).

2.2.3. Biochemical and molecular characterisation of bacterial isolates

Gram stains as well as a KOH tests were done on the Nine bacterial isolates selected from the *in vitro* screening for greenhouse studies. Two of the isolates tested negative for the KOH test and stained Gram negative while the other seven tested positive for both tests (Table 2.4). PCR products with expected sizes that were sent for sequencing analysis showed that four of the nine isolates were identified as *Bacillus* species (B8, B57, M1 and S6), two as *B. amyloliquefaciens* (B7 and I120) and one isolate was identified as *B. cereus* (I48). The other two isolates were identified as *Pseudomonas* species (I124 and I74) (Table 2.4).

Table2.4. Blast and identification details of the selected bacterial isolates for greenhouse studies

Isolate No.	Isolate name	Identified Species	Primer	E-value	% Similarity	Accession Number	Gram Stain	KOH Test
1	B7	*Bacillus amyloliquefaciens*	16S rRNA	0.0	100%	KJ501094.1	+	+
2	B8	*Bacillus* species	16S rRNA	-	88-90%	-	+	+
3	B57	*Bacillus* species	16S rRNA	-	> 95%	-	+	+
4	M1	*Bacillus* species	16S rRNA	0.0	100%	KJ604990.1	+	+
5	S6	*Bacillus* species	16S rRNA	0.0	100%	KJ604990.1	+	+
6	I48	*Bacillus cereus*	16S rRNA	0.0	100%	KJ534497.1	+	+
7	I74	*Pseudomonas* species	16S rRNA	0.0	100%	GQ375795.1	-	-
8	I120	*Bacillus amyloliquefaciens*	16S rRNA	0.0	100%	KJ501094.1	+	+
9	I124	*Pseudomonas* species	16S rRNA	-	98%	Cp007012.1	-	-

2.3. Discussion

The aim of this study was to isolate, screen and identify bacterial isolates antagonistic to *Pyricularia grisea*. Two *Trichoderma* formulation *T*. *harzianum* -77 and *T*. *harzianum* kd were also screened for their ability to suppress *P. grisea in vitro*. A total of 87 bacterial isolates were screened for their ability to suppress *P. grisea* in an *in vitro* dual culture assay. Of the 87, 18 of the isolates demonstrated antagonistic activity against *P. grisea*. During the secondary screening test, nine isolates repeatedly showed potential as biological control agents of *P. grisea*. The isolates were identified either as *Pseudomonas species* or *Bacillus* species based on the sequencing of the 16S rRNA gene. The *Trichoderma* strains also showed a strong antagonistic effect towards *P. grisea*.

A dual culture assay is extensively used as one of the *in vitro* tests for the preliminary screening and selection of bacterial biological control agents. Antagonistic effect against *P. grisea* was confirmed by limited growth or the complete absence of fungal mycelium in the inhibition zone between the fungus and the antagonistic bacteria. The zones of inhibition are evidence of the production of either antibiotics, toxic metabolites including diffusible and volatile metabolites or lytic enzymes, all of which are recognized mechanisms for biological control (Karimi *et al*., 2012; Li *et al*., 2012; Zhao *et al*., 2013; Song *et al*., 2014). These compounds may dissolve the cell wall of the pathogen mycelium and block normal growth. Several authors have reported the inhibition of *P. grisea* growth by antifungal metabolites produced by *Bacillus* and *Pseudomonas* species (Viji *et al*., 2003; Karthikeyan and Gnanamanickani 2007; Tendulkar *et al*., 2007; Naureen *et al*., 2009; Shan *et al*., 2013; Suryadi *et al*., 2013; Spence *et al*., 2014). The appearances of brown pigmentation of the fungal colonies were observed in the presence of the antagonists, except for Isolate I48. The modification and appearance of the fungal mycelium might be due to the production of antifungal secondary metabolites. Idris *et al*. (2007), El Hassni *et al*. (2007) and Ziam *et al*. (2013) also reported modification of mycelium appearance due to antifungal secondary metabolite production. *Bacillus* and *Pseudomonas* species produce different antifungal metabolites that might be used as a primary mechanism in suppressing plant disease (Sansinenae and Ortiz 2011; Haddad *et al*., 2013; Spago *et al*., 2014).

The taxonomic identification of biological control agents was done by molecular biological methods. The sequencing of the 16S rRNA region is used widely to identify bacterial microbial organisms (Kim *et al*., 2012). In this study it was observed that identification based on the 16S rRNA gene sequencing was limited and only sufficient to discriminate and characterise two of the nine selected bacterial isolates to species level. The 16S rRNA gene sequencing lacks resolution when dealing with highly related species (Ramette *et al*., 2011; Solanki *et al*., 2012). Successful classification of the genus *Pseudomonas* can be achieved by using the conserved protein-coding housekeeping genes *gyr*B, *rpo*D and *rpo*B (Parkinson *et al*., 2011). Solanki *et al.* (2011) found that by using polyphasic genotypic fingerprinting tools (ERIC and BOX PCR) they were able to distinguish intra-species variability among *Bacillus* strains.

The *T. harzianum* strains inhibited the growth of *P. grisea*. These results are in accordance with those reported by Gouramans (1995), Ouazzan *et al*. (1998); Hajano *et al*. (2012) and Consolo *et al*. (2012), who observed that *T. harzianum* caused >80% mycelial growth inhibition of *P. grisea in vitro*. *Trichoderma harzianum* strains are well known producers of secondary metabolite and cell wall degrading enzymes that can be implicated in inhibition of radial growth of many pathogenic fungi (Abeysinghe, 2007). Other mechanisms involved in *Trichoderma* antagonism which were not studied in this work include competition for space and nutrients and mycoparasitism whereby the *Trichoderma* directly attacks the pathogen by exerting lytic enzymes such as chitinases and proteases (Bae *et al*., 2011; Lopez-Monejar *et al*., 2011; Kamala and Devi, 2014).

The bacterial isolates and the *T. harzianum* strains used in this study showed potential as biocontrol agents against *P. grisea*. They were able to suppress the growth of the fungus *in vitro*. The assumed mode of action used by the bacterial isolates is antibiosis and the production of other extra-cellular metabolites because of observed clear zones of inhibition and colour changes in fungal mycelium. However, there is a possibility that other mechanisms may play a role in the antagonistic behaviour of the biocontrol agents. Therefore, additional experiments need to be conducted in order to determine the mode of action involved in the biocontrol activity of these agents.

Bacillus and *Pseudomonas* spp have been successfully used against foliar pathogens. In a study by Maachia *et al.* (2015), foliage of potted grapevine plants infected with powdery mildew and *Botrytis cinerea* were treated with suspensions of *B. subtilis* B27 and B29 strains. The *B. subtilis* B27 and B29 strains were able to effectively reduce powdery mildew up to 50% and 60 %, respectively. Also*, B. subtilis* B27 and B29 decreased *B. cinerea* development on grape leaf by 77% and 99%, respectively. In another study by Li *et al.* (2012), they evaluated the biological control effect of *B. subtilis* strain E1R-j strain on *Puccinia striiformis* fsp *tritic*i (*Pst*) development on wheat using bacterial suspensions (BCS) and fermentation liquid with and without bacterial cells (FLBC and FL). Under greenhouse conditions, foliar application of FLBC and FL 24 h and 0 h before *Pst* inoculation significantly reduced disease severity. The control efficacy was between 54.0% and 87.7%. With formulated BCS, only the application 24 h before *Pst* inoculation significantly reduced rust severity with a control efficacy of 51.8%. Greenhouse experiments done by Purohit et al (2013) using *Pseudomonas* spp (psf-28 and psf-11) against *Gloeocercospora sorghi* on sorghum showed maximum reduction in disease severity by psf-28 (45.05%) and psf-11 (40.33%) with 3 foliar treatments. The treatments were given as foliar sprays 35, 45 and 55 days after sowing. These results demonstrate that *Bacillus* and *Pseudomonas* spp have the potential to control foliar diseases under greenhouse conditions.

The results in this study were useful in identifying potential candidates for the biocontrol of *P. grisea* on ryegrass. These isolates were subsequently tested under greenhouse conditions to ascertain their effectiveness in controlling *P. grisea in planta*.

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CHAPTER THREE

In vivo screening of biological control agents against gray leaf spot of ryegrass under greenhouse conditions

Abstract

Greenhouse studies were conducted to evaluate the efficacy of nine bacterial isolates on *Pyricularia grisea*, the causal organism of gray leaf spot on annual (*Lolium multiflorum* L.) and perennial (*Lolium perenne* L.) ryegrass. The bacterial isolates were previously selected based on their ability to suppress mycelia growth of *P*. *grisea* during *in vitro* studies. Two commercially available *Trichoderma* formulations, *Trichoderma harzianum* strain kd (T.kd) and *Trichoderma harzianum* strain B77 (T.77), and the fungicide azoxystrobin were also tested. In Experiment 1 conducted on annual ryegrass, two bacterial isolates, *Bacillus* spp. S6 and B57, reduced gray leaf spot (GLS) severity by 26.0 and 34.0%, respectively. In Experiment 2 conducted on annual ryegrass, *Bacillus* sp. M1 reduced disease severity by 20%; *B. amyloliquefaciens* 120 and *Pseudomonas* sp. I74 both reduced GLS by 19.0%. However, reduction in GLS severity in both experiments was not significant at $P > 0.05$. *Trichoderma harzianum* T.kd and T.77 both caused little reduction in GLS severity. Azoxystrobin reduced GLS severity by 28.0% in Experiment 1 while in Experiment 2 it reduced GLS by 32.0%. In Experiments conducted on perennial ryegrass, *B.cereus* I48 and *Bacillus* sp. S6 consistently reduced GLS severity by 35.0 and 30.0%, respectively, in Experiment 1 and by 41.0% and 39.0%, respectively, in Experiment 2. Azoxystrobin caused a significant reduction in GLS severity by 35.0% in Experiment 1 and 54.0% in Experiment 2. *Trichoderma* formulations T.kd and T.77 did not significantly reduce GLS severity in either experiment. The best control was recoded with *B.cereus* I48 on perennial ryegrass which consistently reduced GLS severity by 35.0% to 42.0%in Experiment 1 and 2 respectively. The best performing isolate, bacillus spp. M1, on annual ryegrass was selected from Experiment 2, reducing GLS severity by 20%. The selected biocontrol agents have the potential to control GLS disease and were used in field experiments (Chapter 5).

3.1. Introduction

The successful management of gray leaf spot (GLS) is highly dependent on chemical fungicides (Ma, 2006). Quinone outside inhibitor (Q_OI) fungicides such as azoxystrobin, trifloxystrobin and pyraclostrobin are among the most effective fungicides for the control of GLS (Ma, 2006). The main mechanism of action by these fungicides is the inhibition of mitochondrial respiration of the pathogen (Bagi *et al*., 2014). They also negatively influence mycelial growth, sporulation and spore germination of several fungi. The development of resistance is a concern with the use of Q_OI fungicides due to their single-site mode of action. Heavy reliance on the fungicide runs the risk of selecting fungicide-resistant strains of *Pyricularia grisea*. There have been reports of resistance development of azoxystrobin within the pathogen population on the following hosts: perennial ryegrass (Vincelli and Dixon, 2002; Kim et al., 2003) and wheat (*Triticum aestivum* L.) (Castroagudin *et al*., 2015; Oliveira *et al*., 2015).

Managing fungal disease of plants with bacterial antagonists either as an alternative or supplement to fungicides could be of great benefit (Anand, 2010). The genus *Bacillus* represent a major source of microbial biological control agents (Pane and Zaccardelli, 2015). *Bacillus* species are able to form heat and desiccation-resistant spores that allow them to resist adverse environmental conditions and permit easy formulation and storage of commercial products (Tan *et al.,* 2013). Being able to adapt to extreme conditions makes *Bacillus* spp widespread in various environments, thus making them versatile in biocontrol application (Pane and Zaccardelli, 2015). Several *Bacillus* biological control agents have shown consistent suppression of foliar diseases, for example, rice sheath blight (Chumthong *et al*., 2015), Asian soybean rust (Dorighello *et al*., 2015) and grapevine diseases, grey mold and powdery mildew (Maachia *et al*., 2015) under controlled environments.

Pseudomonas is a genus of Gram-negative bacteria. *Pseudomonas* spp a diverse group of microorganisms that occupy many different niches (Singh *et al*., 2015). They have great potential as biological control agents (Gao *et al*., 2012). This group of organisms is known to produce a wide range of antifungal metabolites (Gao *et al*., 2012, Abraham *et al*., 2013). Some well-known non-pathogenic biocontrol agents

from the genus include: *P. aeruginosa*, *P. fluorescens*, *P. chlororaphis*, *P. stutzeri* and *P. putida* (Goswami *et al*., 2015).

In this chapter nine bacterial isolates previously selected from *in vitro* screening studies (Chapter Two) for antagonistic activity against *P. grisea* were tested on annual and perennial ryegrass under greenhouse conditions for their ability to suppress gray leaf spot.

3.2. Materials and methods

3.2.1. Source of seed

The perennial ryegrass seed *Lolium perenne* L. (cv. Arrow) was provided by Barenbrug Seeds (Pty) Ltd, Cape Town, South Africa. The annual ryegrass seed *Lolium multiflorum* L. (cv. Barrextra) was supplied by Farmers Agri-Care (Pty) Ltd, Howick, South Africa.

3.2.2. Inoculum preparation of biocontrol agents

(a) Bacterial isolates

The bacterial isolates used in this study were selected from *in vitro* screening studies for antifungal activity against *P. grisea* (Chapter Two). Fresh cultures were prepared from frozen stock cultures by sub-culturing the individual isolates on nutrient agar (NA) plates and incubated at 28°C for 24 h. The bacterial cultures were re-suspended in a 1.5% wetting agent, Break-Thru® solution (Universal Crop Protection (Pty) Ltd., Kempton Park, South Africa). Cell density was determined using a Helber Bacterial Counting Chamber (Paul Marienfield-superior GmbG & Co KG) and the concentration of 10^8 cells ml^{-1} was established.

(b) Fungal isolates

Trichoderma harzianum strain kd (T.kd) and *T. harzianum* strain B77 (T.77) were used throughout the experiments. T.kd and T.77 are registered biocontrol products provided by Plant Health Products (Pty) Ltd, Nottingham Road, South Africa. The T.77 and T.kd treatments were prepared by suspending 2g and 1g, respectively, of the product into a liter of tap water, as recommended by the manufacturer, making up a suspension containing $1x10^9$ conidia ml^{-1}.

3.2.4. Inoculum production of *Pyricularia grisea* and inoculation of plants

An isolate of *P. grisea* was grown on oat meal agar (OMA) for 7-14 d. Agar blocks containing actively growing mycelia were transferred to OMA plates and incubated in the dark at 28°C for 7-14 d. The fungal plates were then placed under continuous white light at 20°C for 7-14 d in order to induce sporulation. Using a scalpel, the mycelia were removed from the culture by scraping the surface of the plates. The conidia were harvested and suspended in a 1.5% Break-Thru® solution. Using a haemocytometer, the final concentration of conidia was adjusted to approximately $1x10^5$ conidia ml^{-1}.

3.2.5. Screening of selected biocontrol agents against *Pyricularia grisea* under greenhouse conditions

(a) Plant Material

Perennial ryegrass cv. Arrow and annual ryegrass cv. Barrextra were grown in 15cm diameter plastic pots filled with compost pine bark growth medium (Gromor, Cato Ridge, South Africa). Plants were individually irrigated as required from the first day of sowing until maturity. The irrigation water contained NPK Easy Grow Starter Fertiliser 2.1.2 (43) (Ag-Chem Africa (Pty) Ltd, Pretoria, South Africa) applied at 1 g l^{-1} of nutrient solution a week after germination. The plants were then fertilised once a week from then onwards. The plants were kept in the greenhouse under controlled environmental conditions with relative humidity at >80% during the day and the temperature set at 27±1°C during the day and 18°C at night.

(b) Inoculation of plants

The greenhouse experiments were carried out at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. Pots of four-week old ryegrass plants were each treated separately with the following: (1). bacterial antagonists (10^8 cells ml^{-1}), (2). T.kd ($1x10^9$ conidia ml^{-1}), (3). T.77 ($1x10^9$ conidia ml^{-1}) and (4). Amistar® (azoxystrobin) (600 ml L^{-1}). The biological control agents were applied as foliar applications using a 500 ml hand spray bottle. Two days after treatment, the plants were challenge-inoculated with *P. grisea* ($1x10^5$conidia ml^{-1}) by spraying the leaves until run-off. The fungicide was applied as soon as disease symptoms on the ryegrass were observed. Plants sprayed with only the 1.5% Break-Thru® solution served as the Uninoculated Control. After pathogen inoculation, polyethylene bags were placed over each inoculated plant to create high humidity. The polyethylene

bags were removed after 48 hr. All treatments were replicated three times. The plants were arranged in a randomised complete blocks design. Disease severity ratings were made 7 d after pathogen inoculation and every week thereafter for six weeks.

(c) Disease assessment

Gray leaf spot severity was rated visually on a scale of 0 to 10, where 0 = no disease; 1= 1-10%of whole plant area necrotic and blighted; 2= 11-20%; 3= 21-30%; 4= 31-40%; 5= 41-50%; 6= 51-60%; 7= 61-70%; 8= 71- 80%; 9= 81-90%; 10= 91-100%. The mid-point values were used to calculate the Area Under the Disease Progress Curve (AUDPC) for all treatments (Shaner and Finney, 1977) before statistical analysis.

3.2.6. Data analysis

The AUDPC values and the final disease severity values (arcsine transformed) were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc, 2011). Treatment means were separated using Duncan multiple range test at the 5% probability level (P = 0.05).

3.2. Results

3.3.1. Effects of selected biological control agents on the control of *Pyricularia grisea* on annual ryegrass under greenhouse conditions

In Experiment 1, seven bacterial isolates reduced GLS severity with a percentage reduction ranging from 6.2 - 34.2% (Table 3.1). *Bacillus* spp B57 caused the greatest reduction (34.2%) followed by *Bacillus* spp S6 (26.4%). The *T. harzianum* formulations caused little reduction in disease severity, with T.77 causing a 6.2% reduction in GLS severity while T.kd gave no disease reduction compared to the pathogen inoculated control. Azoxystrobin reduced GLS severity by 28.3%. All treatments had no significant effect on reduction of GLS severity (P = 0.49) (Table 3.1). The AUDPC values were also not significantly different among the treatments (P = 0.35).

In Experiment 2, eight bacterial isolates reduced GLS severity with a disease reduction ranging from 1.9 - 20.9% (Table 3.1). The isolates causing the greatest disease reduction were *Bacillus* spp M1 (20.9%), *B. amyloliquefaciens* I120 and *Pseudomonas* spp I74 both with 19.0%. These were not significantly different from

each other (P = 0.07). T.kd and T.77 reduced GLS severity by 1.9 and 7.6% respectively. Among the treatments the fungicide was the most affective with a 32% reduction of GLS severity (Table 3.1). The AUDPC values were significantly different among the treatments (P = 0.03) (Table 3.1).

3.3.2. Effects of selected biological control agents on the control of *Pyricularia grisea* on perennial ryegrass under greenhouse conditions

In Experiment 1, all bacterial isolates caused a reduction in GLS severity between 5.2–35.6% (Table 3.2). The AUDPC were significantly different among the treatments (P = 0.001). *Bacillus* spp M1, *B. cereus* I48 and *B. amyloliquefaciens* B7 caused the greatest reduction in GLS severity, reducing disease severity significantly by 35.6% (P= 0.001). The bacterial isolates also caused significantly lower AUDPC values. Percentage reduction in GLS severity caused by azoxystrobin was also 35.6 %. T.kd and T.77 both reduced GLS severity by 5.2%. This was not significant (P = 0.53).

In Experiment 2, all the bacterial isolates caused a reduction in disease severity ranging from 12.3 – 49%. *Pseudomonas* spp I74 and *B. cereus* I48 significantly reduced disease severity by 41.8% (P = 0.02) and 49.1% (P = 0.009), respectively. The fungicide control significantly reduced GLS severity by 54.3% (P = 0.003). The *Trichoderma* formulations both reduced GLS severity, but not significantly, by 26.4 % (P = 0.17). *Pseudomonas* spp. I74 (565.8), *B. cereus* I48 (714.2) and azoxystrobin (624.2) caused significant reduction in AUDPC values (P < 0.05). The AUDPC values for the *Trichoderma* formulations T-kd and T.77 were 869.1 and 1160.8, respectively. Although the AUDPC value of T-kd was lower than that of T.77, there was no significant difference between the two (P = 0.8; P = 0.2).

Table 3.1 Effects of different treatments on disease severity of gray leaf spot caused by *P. grisea* on annual ryegrass cv Barrextra

Treatment	Experiment I			Experiment II		
	Foliar disease severity[1,2,3]	**% reduction in GLS severity**	**AUDPC[3,4]**	**Foliar disease severity[1,2,3]**	**%reduction in GLS severity**	**AUDPC[3,4]**
Pyricularia grisea	48.5ab	-	1172.5ab	52.5ab	-	1359.2a
Bacillus species (M1)	45.5ab	6.2	985.8ab	41.5abc	20.9	915.8bc
Bacillus cereus (I48)	42.5ab	12.3	1020.8ab	51.5ab	1.9	1160.8abc
Bacillus species (S6)	35.7ab	26.4	892.5b	55.4ab	-5.7	1184.2abc
Pseudomonas species (I124)	45.5ab	6.2	1102.5ab	45.5abc	13.2	939.2bc
Bacillus amyloliquefaciens(B7)	41.5ab	14.4	962.5ab	48.5abc	7.6	1166.5abc
Bacillus species (B8)	52.5ab	-8.3	1219.2ab	45.5abc	13.2	962.5bc
Bacillus species (B57)	31.8b	34.2	857.5b	42.5abc	7.6	1067.5abc
Bacillus amyloliquefaciens(I120)	42.5ab	12.3	1125.8ab	42.5abc	19.0	904.2c
Pseudomonas species (I74)	55.4a	-14.4	1650.8a	48.5abc	19.0	834.2c
Azoxystrobin	34.7ab	28.3	1359.2ab	35.7c	32.0	927.5bc
T. harzianum-kd (T.kd)	52.5ab	-8.3	1382.5ab	51.5ab	1.9	1277.5ab

T. harzianum-77 (T.77)	45.5ab	6.2	1335.6ab	48.5abc	7.6	927.5bc
F – ratio	0.99		1.18	1.96		2.37
P – value	0.49		0.35	0.07		0.03
CV%	16.32		31.32	9.12		17.72

[1]Visual ratings of foliar disease severity (0 – 100). Numbers are arcsine transformed.
[2] The first visual ratings made on whole plant at 1 week after inoculation with *Pyricularia grisea.* Rating were made weekly thereafter for 6 weeks [1 = 0%; 2 = 1 – 10%; 3 = 11 – 20%; 4 = 21- 30%; 5 = 31 – 40%; 5 = 41- 50%; 6 = 51- 60%; 7 = 61 – 70%; 8 = 71 – 80%; 9 = 81 – 90%; 10 = 91 – 100%]
[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)
[4]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates

Table 3.2. Effects of different treatments on disease severity of gray leaf spot caused by *P. grisea* on parenniall ryegrass cv Arrow

Treatment	Experiment I			Experiment II		
	Foliar disease severity[1,2,3]	**% reduction in GLS severity**	**AUDPC[3]**	**Foliar disease severity[1,2,3]**	**%reduction in GLS severity**	**AUDPC[3,4]**
Pyricularia grisea	55.4a	0	1557.5ab	48.5a	0	1140.8ab
Bacillus species (M1)	35.7d	35.6	781.1dce	35.6abc	26.4	857.5abcd
Bacillus cereus (I48)	35.7d	35.6	798.0dce	28.2abc	41.8	714.2abcd
Bacillus species (S6)	38.6bcd	30.3	962.5dce	29.1abc	39.9	694.2bcd
Pseudomonas species (I124)	38.6bcd	30.3	892.5dce	35.6abc	26.4	869.2abcd
Bacillus amyloliquefaciens(B7)	35.7d	35.6	740.8dce	35.6abc	26.4	997.5abcd
Bacillus species (B8)	45.5abc	17.8	962.5dce	42.5ab	12.3	1032.5abc
Bacillus species (B57)	42.5bcd	23.3	1020.8dce	35.6abc	26.4	880.8abcd
Bacillus amyloliquefaciens (I120)	48.5abc	12.5	915.8dce	35.6abc	26.4	834.2abcd
Pseudomonas species (I74)	58.5ab	5.2	1289.2bc	24.6bc	49.1	565.8bcd
Azoxystrobin	35.7d	35.6	1009.0dce	22.1c	54.3	624.2bcd

T. *harzianum*-kd (T.kd)	52.5ab	5.2	1172.5dc	35.6abc	26.4	869.1abcd
T. *harzianum*-77 (T.77)	52.5ab	5.2	1641.2a	41.5ab	14.4	1160.8a
F – ratio	4.19		6.30	1.57		1.54
P – value	0.001		0.001	0.17		0.11
CV%	9.04		18.56	17.51		27.56

[1]Visual ratings of foliar disease severity (0 – 100). Numbers are arcsine transformed.
[2] The first visual ratings made on whole plant at 1 week after inoculation with *Pyricularia grisea.* Rating were made weekly thereafter for 6 weeks [1 = 0%; 2 = 1 – 10%; 3 = 11 – 20%; 4 = 21- 30%; 5 = 31 – 40%; 5 = 41- 50%; 6 = 51- 60%; 7 = 61 – 70%; 8 = 71 – 80%; 9 = 81 – 90%; 10 = 91 – 100%]
[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)
[4]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates

3.4. Discussion

Greenhouse experiments showed inconsistencies in the performance of the bacterial antagonists applied as foliar treatments on annual ryegrass for the control of gray leaf spot. In Experiment 1 *Bacillus* spp S6 and *Bacillus* spp B57 caused the greatest reduction in GLS severity. However, in Experiment 2, *Bacillus* spp M1, *Pseudomonas* spp I74 and *B. amyloliquefaciens* 120 caused the greatest reduction in GLS severity. Reduction in GLS severity caused by the bacterial antagonists was not significant in either experiment. In the experiments conducted on perennial ryegrass, *B. cereus* I48 and *Bacillus* spp S6 significantly reduce the AUDPC values and GLS severity. The performances of the isolates were consistent in both experiments. Neither of the *Trichoderma* strains, T.kd and T.77, satisfactorily controlled GLS on both the annual and perennial ryegrass cultivars, whereas azoxystrobin consistently reduced AUDPC values and GLS severity in both annual and perennial ryegrass.

The use of bacteria as biocontrol products is of interest in crop protection. In a study by Raham *et al*. (2015) live *B. amyloliquefaciens* cells and solid phase extraction (SPE)-enriched surfactin were applied to perennial ryegrass using a root-drench application. Both significantly reduced disease incidence and disease severity of GLS on perennial ryegrass. Suppression of *P. grisea* was as a result of induced systemic resistance. Viji *et al*. (2002) isolated three biocontrol isolates of *Pseudomonas aeruginosa* (B12, B15 and B35) from spent mushroom substrates. These isolates were applied as foliar treatment at different intervals on perennial ryegrass maintained in controlled environment chambers. Isolates B12, B15 and B38 significantly suppressed GLS, regardless of the application interval. In this study we were able to show that *B. cereus* I48 and *Bacillus* spp S6 significantly reduced AUDPC values and GLS severity on perennial ryegrass. This study confirms that biological control agents have the potential to suppressing GLS on perennial ryegrass.

To the best of our knowledge this is the first study on the biological control of GLS on annual ryegrass. However, bacterial isolates have been successfully used against *Pyricularia grisea* on rice (*Oryza sativa* L.) (Sutyadi *et al*., 2013; Meng *et al.,* 2015) and perennial ryegrass. The bacterial isolates tested on annual ryegrass were inconsistent

in their performance compared to the results obtained from the perennial ryegrass experiments. Several factors may have contributed to the inconsistent performances, including fluctuations in the greenhouse temperatures, the mode of application of the bacterial antagonist, and the pathogen inoculum level may have been too high. The inherent genetic variability between the cultivars also may have contributed to the variable responses observed.

In their study, Viji *et al*. (2002) also found that disease severity and disease incidence for all three biocontrol isolates used were not significantly different from that of the fungicide, propiconazole, and in one experiment *P*. *aeruginosa* Isolate B12 was not significantly different from azoxystrobin in reducing disease severity. In this study, we demonstrated that the levels of GLS control provided by the biocontrol agents *B. cereus* I48 and *Bacillus* spp S6 were comparable to that provided by azoxystrobin on perennial ryegrass.

In order to be effective, biological control agents must grow and proliferate. Therefore, effective antagonists must become established and be able to maintain active populations against pathogens during periods of plant infection (Lo *et al*., 1997). *Trichoderma harzianum* has been used as a foliar treatment for several plant diseases. These include rice brown spot (Khalili *et al*., 2012), early blight of tomato (Ramanujam *et al*., 2015) and wheat leaf rust (El-Sharkawy *et al*., 2015). In a study by Lo *et al*. (1997), spray application of *T. harzianum* strain 1295-22 resulted in disease suppressive population levels on leaves that were sufficient to suppress brown patch and dollar spot. T. *harzianum* strain 1295-22 was able to survive on turf leaves for at least four weeks in a growth chamber. However, in this study, T.kd and T.77 were not able to significantly reduce GLS on ryegrass.

In this study we also demonstrated that *B. cereus* I48 and *Bacillus* spp S6 were able to control GLS on perennial ryegrass. Furthermore, these isolates provided levels of disease control that was comparable to the fungicide azoxystrobin. These isolates will further be tested under field conditions. Some bacterial antagonists reduced GLS on annual ryegrass, although not significantly, and these isolates were inconsistent in their

performance. *Trichoderma* T.kd and T.77 did not significantly reduce GLS severity on both annual and perennial ryegrass.

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CHAPTER FOUR

Efficacy of various concentrations and application frequencies of potassium silicate against gray leaf spot of ryegrass under greenhouse conditions

Abstract

Four different concentrations (50, 100, 200 and 300 ppm) of a liquid formulated potassium silicate (KSil) were evaluated for their efficacy in controlling gray leaf spot on annual and perennial ryegrass under greenhouse conditions. The potassium silicate solution was applied as a drench once or twice week. In the experiments conducted on perennial ryegrass, three KSil concentrations, 100 ppm:1 (applied once a week), 200 ppm:2 (applied twice a week) and 300 ppm:1 (applied once a week), significantly reduced GLS severity by 17.0, 19.3 and 23.5%, respectively, ($P = 0.02$; $P = 0.006$; $P = 0.0007$). In the experiments conducted on annual ryegrass, the 100 ppm:2 (applied twice a week) and the 300 ppm:1 (applied once a week) concentrations both significantly ($P = 0.01$) reduced GLS severity by 27.3% and the 200ppm:1 (applied once a week) concentration significantly reduced GLS severity by 29.8% ($P = 0.009$). Overall, the KSil concentration, 300 ppm:1 (applied once a week) and the 200ppm:1 (applied once a week) were the most effective in reducing GLS severity on annual and perennial ryegrass respectively. These concentrations were therefore selected for field experiments (see Chapter Five).

4.1. Introduction

Nutrient management strategies serve as an alternative approach in managing plant diseases and reducing the use of fungicides (Lemes *et al*., 2011). Mineral nutrients play an important role in the physiological functioning of plants and have been shown to be an important component for managing plant diseases (Read and Pratt, 2012). Silicon (Si) is the most abundant element in soils (Epstein, 1999). Although Si has not been proven to be an essential element for plant growth and development, its beneficial role

in stimulating plant growth, grain yield and resistance to abiotic and biotic stress have been documented (Waraich *et al*., 2011; Guntzer *et al*., 2012).

Silicon amendments have been proven to have positive effects on various turf grasses especially in suppressing leaf diseases. Silicon amendments have been reported to suppress gray leaf spot on perennial ryegrass (*Lolium perenne* L.) (Nanayakarra *et al*., 2005; Nanayakarra *et al.,* 2008; Raham *et al*., 2015), powdery mildew on Kentucky bluegrass (*Poa pratensis* L.), gray leaf spot on St. Augustine grass (*Stenotaphrum secundatum* (Waltz) Kuntze) (Brecht *et al*., 2005; Brecht *et al*., 2007) dollar spot and brown patch on creeping bentgrass (*Agrostis palustris* Huds. A.) (Uriate *et al*., 2004) and leaf spot/melting out disease caused by *Bipolaris cynodontis* on Bermuda grass (*Cynodon dactylon* L. Pers.) (Datnoff and Rutherford, 2004).

There are two mechanisms by which Si confers disease suppression: (i) Deposition of Si on tissue surfaces, which acts as a physical barrier. This prevents or delays fungal penetration of the epidermal cells and makes the plant less susceptible to enzymatic degradation by fungal pathogens; (ii) Silicon also functions as a signal to increase the activities of defense enzymes and the rapid transcription of genes associated with plant resistance. It is also responsible for the enhanced induction of the production of phenolics which act as signal molecules or antioxidants therefore inducing resistance. Silicon also enhances the production of lignin which is deposited in secondary cell wall of plants and acts as a physical barrier against pathogen invasion, and the production of phytoalexins which are low molecular weight antimicrobial compounds (Sun *et al*., 2010; Guntzer *et al*., 2012; Van Bockanhen *et al*., 2013; Jeandet *et al*., 2013; Fotoohiyan *et al*., 2015; Malinovsky *et al*., 2015).

Accumulation of silicon in plant tissues varies among different plant species. It ranges from 1-100 g kg^{-1} of plant dry weight (Ma and Takahashi, 2007). There are three possible types of Si uptake in higher plants: active, passive and rejective (Takahashi *et al*., 1990). In some graminaceous plants such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) (Van der Vorn, 1980; Jarvis, 1987), barley (*Hordeum vulgare* L.) (Barber and Shone, 1966) and ryegrass (Jarvis, 1987), Si is taken up actively. Si tissue

concentration can also vary among genotypes of the same species as demonstrated in rice (Deren, 2001; Ma *et al*., 2007), sugarcane (*Saccharum officinarum* L.) (Deren, 2001) and barley (Ma *et a*l., 2003)

The aim of this study was to investigate the efficacy of different concentrations of liquid formulation of potassium silicate and application frequencies on gray leaf spot of annual and perennial ryegrass grown under greenhouse conditions.

4.2. Materials and Methods

4.2.1. Potassium Silicate (KSil)

Silicon was applied as a soluble liquid formulation of potassium silicate (K_2SiO_3), Agrisil K50, (PQ Corporation, South Africa) containing 33 g kg^{-1} potassium and 96 g kg^{-1} silicon.

4.2.2. Source of seed

The perennial ryegrass seed *Lolium perenne* (Arrow) used in this study was provided by Barenbrug Seeds (Pty) Ltd, Cape Town, South Africa. The annual ryegrass seed *Lolium multiflorum* (Barrextra) was supplied by Farmers Agri-Care (Pty) Ltd, Howick, South Africa.

4.2.3. Production of inoculum

An isolate of *P. grisea* was grown on oat meal agar (OMA) for approximately 7-14 d. Agar blocks containing actively growing mycelia were transferred to OMA plates and incubated in the dark at 28°C for 7-14 d. Using a scalpel, the mycelia were removed by carefully scraping the surface of the agar plates. The fungal plates were then placed under continuous light at 20°C for 7-14 d in order to induce sporulation. The conidia were harvested and suspended in distilled water. Using a haemocytometer, a final concentration of the conidia suspension was adjusted to approximately $1x10^5$ conidia ml^{-1}.

4.2.4. Control of *Pyricularia grisea* using different concentrations of potassium silicate applied at varying frequencies

(a) Plant Material

Perennial ryegrass cv. Arrow and annual ryegrass cv. Barrextra were grown in 15 cm diameter plastic pots filled with composted pine bark growth medium (Gromor, Cato Ridge, South Africa). Plants were manually irrigated as required from the first day of sowing until maturity. The irrigation water contained NPK Easy Grow Starter Fertiliser 2.1.2 (43) (Ag-Chem Africa (Pty) Ltd, Pretoria, South Africa) and was applied at 1 g l^{-1} of nutrient solution once a week after germination. The plants were then fertilised once a week. The plants were kept in the greenhouse under controlled environmental conditions with relative humidity at >80% during the day and the temperature set at 27±1°C during the day and 18°C at night.

(b) Effects of different concentrations of potassium silicate applied at varying frequencies

The greenhouse experiments were carried out at the University of KwaZulu-Natal, Pietermaritzburg, South Africa. The treatments were applied as follows: 50 ppm:1 (applied once a week) 50 ppm:2 (applied twice a week), 100 ppm:1 (applied once a week), 100 ppm:2 (applied twice a week), 200 ppm:1 (applied once a week), 200 ppm:2 (applied twice a week), 300ppm:1 (applied once a week) and 300 ppm:2 (applied twice a week) and 0 ppm (Water Control). The plants were drenched separately with each KSil concentration one week after germination. Potassium chloride (KCl) salts were also used as a Positive Control (equivalent levels of potassium only), KCl:1 (applied once a week) and KCl:2 (applied twice week). Four-week old ryegrass plants were inoculated with conidial suspensions by spraying the leaves until run-off using a 500 ml hand held spray bottle. After pathogen inoculation, polyethylene bags were placed over each inoculated plant to maintain high humidity. The polyethylene bags were removed after 48 h. All treatments were replicated three times. The plants were arranged in a randomised complete block design. Disease severity ratings were made 7 d after pathogen inoculation and every week thereafter for six weeks. The experiments were repeated twice.

(c) Disease assessment

Gray leaf spot severity was assessed visually on a scale of 0 to 10, where 0 = no disease; 1= 1-10% of whole plant area necrotic and blighted; 2= 11-20%; 3= 21-30%; 4= 31-40%; 5= 41-50%; 6= 51-60%; 7= 61-70%; 8= 71- 80%; 9= 81-90%; 10= 91-100%. The infected plant area was measured weekly for six weeks. The mid-point values were used to calculate the Area Under the Disease Progress Curve (AUDPC) for all treatments (Shaner and Finney, 1977) before statistical analysis.

3.2.6. Data analysis

The AUDPC values and the final disease severity values (arcsine transformed) were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc., 2011). Treatment means were separated using Duncan Multiple Range Test (DMRT) at the 5% probability level (P = 0.05).

4.3 Results

4.3.1 Effects of different potassium silicate concentrations and frequency of application on gray leaf spot severity on perennial ryegrass under greenhouse

No significant differences were observed in disease severity as a result of the different KSil treatments. No significant difference was observed between the pathogen inoculated Water Control and KCl Control. However, significant (P < 0.05) differences were observed between three KSil treatments and the pathogen inoculated Water Control, with percentage disease reduction between 13 and 23.5% (Table 4.1). The KSil concentrations causing the highest percentage reduction in GLS severity were 100 ppm:1, 200 ppm:2 and 300 ppm:1, which reduced disease severity significantly by 17.0, 19.3 and 23.5%, respectively, relative to the pathogen inoculated Water Control (P = 0.02; P = 0.006; P = 0.0007). The 200 ppm:2 and 300 ppm:1 concentrations also caused significantly lower AUDPC values of 1241.8 and 1300.0, respectively, with an AUDPC value of 1854.2 (P = 0.003; P = 0.008). (Table 4.1).

4.3.2. Effects of different potassium silicate concentrations and frequency of application on gray leaf spot severity on annual ryegrass

No significant differences were observed between the different KSil treatments with regards to disease severity. No significant difference was observed between the

pathogen inoculated Water Control and the KCI Control. However, significant differences ($P < 0.05$) were observed between five of the KSil treatments and the pathogen inoculated Control (Table 4.2). The KSil concentrations that caused the highest reduction in GLS severity were 100 ppm:2 and 300 ppm:1($P = 0.01$ for both), reducing disease severity by 27.3% and 200 ppm:1, significantly ($P = 0.009$) reducing disease severity by 29.8%. Of the three treatments only 200 ppm:1 and 300 ppm:1 caused significantly ($P = 0.005$; $P = 0.01$) lowered AUDPC values of 1213.3 and 1225.0, respectively (Table 4.2).

Table 4.1 Effects of different potassium silicate concentrations and frequency of application on grey leaf spot severity on perennial ryegrass under greenhouse conditions

	Experiment I		
Treatment	**Foliar disease severity[1,2,3]**	**% reduction**	**AUDPC[3,4]**
0 (Water Control)	63.8a	0	1854.2a
KCl:1	59.5ab	6.8	1499.2ab
KCl:2	53.8abc	15.7	1391.0ab
50ppm:1	55.5abc	13.1	1554.3ab
50ppm:2	55.5abc	13.1	1551.7ab
100ppm:1	53.3bc	17.0	1529.5ab
100ppm:2	55.5abc	13.1	1356.0b
200ppm:1	55.5abc	13.0	1424.5ab
200ppm:2	51.5bc	19.3	1241,8b
300ppm:1	48.8c	23.5	1300.8b
300ppm:2	53.8abc	15.7	1297.6b
F-ratio	1.72		1.45
P-value	0.10		0.18
%CV	8.6		23.96

[1]Visual ratings of foliar disease severity (0 – 100). Numbers are arcsine transformed.
[2]The first visual ratings were made on whole plant at 1 week after inoculation with *Pyricularia grisea.* Ratings were made weekly thereafter for 6 weeks. [1 = 0%; 2 = 1 – 10%; 3 = 11 – 20%; 4 = 21- 30%; 5 = 31 – 40%; 5 = 41- 50%; 6 = 51- 60% ; 7 = 61 – 70%; 8 = 71 – 80%; 9 = 81 – 90% ;10 = 91 – 100%]
[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)
AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates

Table 4.2. Effects of different potassium silicate concentrations and frequency of application on grey leaf spot severity on annual ryegrass under greenhouse conditions.

	Experiment I		
Treatment	**Foliar disease severity[1,2,3]**	**% reduction**	**AUDPC[3,4]**
0 (Water Control)	67.2a	0	1907.5a
KCl:1	58.8ab	12.5	1612.6abc
KCl:2	62.2ab	7.4	1706.0abc
50 ppm:1	58.8ab	12.4	1650.8abc
50 ppm:2	63.8ab	5.0	1847.7ab
100 ppm:1	58.8ab	12.4	1586.7abc
100 ppm:2	48.8b	27.3	1265.8bc
200 ppm:1	47.2b	29.8	1143.3c
200 ppm:2	50.5ab	24.8	1213.3c
300 ppm:1	48.8b	27.3	1225.0c
300 ppm:2	50.5ab	24.8	1306.7bc
F-ratio	1.85		2.16
P-value	0.07		0.03
%CV	15.38		30.28

[1]Visual ratings of foliar disease severity (0 – 100). Numbers are arcsine transformed.
[2]The first visual ratings were made on whole plant at 1 week after inoculation with *Pyricularia grisea.* Ratings were made weekly thereafter for 6 weeks. [1 = 0%; 2 = 1 – 10%; 3 = 11 – 20%; 4 = 21- 30%; 5 = 31 – 40%; 5 = 41- 50%; 6 = 51- 60% ; 7 = 61 – 70%; 8 = 71 – 80%; 9 = 81 – 90% ;10 = 91 – 100%]
[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)
AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates

4.4. Discussion

In this study we observed that KSil significantly reduced GLS severity in both annual and perennial ryegrass when compared to the pathogen inoculated Water Control when applied at certain concentrations and frequencies of application. The most effective KSil treatments in reducing GLS severity were 300 ppm:1 and 200 ppm:1 in perennial and annual ryegrass, respectively. The AUDPC values at the respective concentrations were also significantly lower than the pathogen inoculated Water Control.

The results in this study support the findings from several studies where the effects of silicon was shown to suppress various diseases of grass species including perennial ryegrass. In a study by Datnoff *et al*. (2005), the severity of GLS on St Augustine grass was significantly reduced by calcium silicate applied at rates between 0 – 10 t ha^{-1}.They reported that the disease decreased between by 5 and 17% in comparison to the control as the rate of calcium silicate increased. They were also able to show that Si was effective in suppressing leaf spot development on Bermuda grass by 38.9%. Brecht et al (2004) demonstrated that silicon reduced the AUDPC of GLS of St Augustine grass by 7 to 68% compared to the untreated control. In their study silicon was applied as calcium silicate slag in a broadcast manner at a rate of 5t ha^{-1}. Lastly, in a study by Nanayakara *et al*. (2008), two sources of silicon, calcium silicate and wollastonite slag, were applied at rates of between 0 and 10 t ha^{-1} and 0 and 24 t ha^{-1} respectively. They were able to show that silicon reduced GLS severity in perennial ryegrass and that both sources of Si reduced disease severity equally.

The probable mechanism of KSil against GLS in this study was through the priming of defence-related enzymes and phenolics associated with plant defense systems. Rahman *et al*. (2015) observed that perennial ryegrass plants inoculated with *P. grisea* and grown in soil amended with Si exhibited greater levels of peroxidase and polyphenol oxidase than those detected in the inoculated control plants grown in soil without Si treatment. They also observed that several phenolic acids including chlorogenic acid and flavonoids, and related expression of genes encoding phenylalanine, ammonia lyase and lypoxygenase were significantly increased in plants treated with Si. In additional studies they also found that Si played a role in perennial

ryegrass defence responses by delaying entrance and colonisation of *P. grisea*. They observed cell-wall apposition, where they found enhanced levels of several defense related compounds including callose, phenolic autofluorogens and lignin-associated polyphenolic compounds in ryegrass grown in Si-amended soils. These compound are known to delay pathogen entry and colonisation in plants. Disease rating scores in plants treated with potassium silicate were much lower 3 weeks into treatment compared to the pathogen inoculated water control.

A KCl control was included in this study to establish whether K played any role in the defensive role of KSil. Our results showed that there were no significant differences between the KCl control and the pathogen inoculated Water Control. Therefore, we can conclude that the reduction in disease severity in annual and perennial was as a result of silicon in potassium silicate.

In conclusion, the KSil concentration and frequency of application that showed the greatest potential to reduce GLS severity in ryegrass were 200 ppm:1 and 300 ppm:1 in annual and perennial ryegrass, respectively. These treatments will be tested under field conditions as part of an integrated disease management strategy for GLS on ryegrass.

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CHAPTER FIVE

Management of gray leaf spot of ryegrass using biological control agents and potassium silicate under field conditions

Abstract

Bacillus cereus I48, *Bacillus* spp M1, *Trichoderma harzianum* strain B77 (T.77), azoxystrobin, potassium silicate (KSil) and the combined application of biological control agents and KSil were evaluated for their efficacy against gray leaf spot (GLS) on perennial ryegrass (*Lolium perenne* L.) and annual ryegrass (*Lolium multiflorum* L.) in the field. None of the combined treatments significantly ($P > 0.05$) reduced GLS severity in both perennial and annual ryegrass when compared to the pathogen inoculated control. The application of KSil and T.77 alone were not effective in controlling GLS on perennial and annual ryegrass. The application of *B. cereus* I48 on perennial ryegrass and *Bacillus* spp (M1) on annual ryegrass resulted in reductions of 36.6.0% and 22.0% in GLS severity, respectively. Significant differences ($P = 0.03$) were only observed between *Bacillus* spp M1 and the pathogen inoculated control. Azoxystrobin significantly reduced GLS severity by 53.6.0% and 44.0% on perennial ryegrass and annual ryegrass, respectively ($P = 0.02$; $P = 0.0002$). Azoxystrobin was able to consistently reduce GLS severity on both annual and perennial ryegrass in the field. *B. cereus* I48 and *Bacillus* spp. M1 showed potential for contributing to the reduction of GLS on ryegrass in the field. Azoxystobrin consistently reduced GLS severity in the field on ryegrass. These results warrants further investigation for the possibility of using the biocontrol agents and chemical control as part of an integrated pest management strategy.

5.1. Introduction

Perennial ryegrass (*Lolium perenne* L.) and annual ryegrass (*Lolium multiflorum* Lam.) are important in forage/ livestock production. Perennial ryegrass can also be used as an amenity grass in sport courses and residential areas (Brazauskas *et al*., 2013). These grasses have superior nutritional and agronomic qualities (Rahman *et al* 2015).

Diseases affecting these grasses cause significant production losses. One such diseases is gray leaf spot (GLS), which is a foliar disease caused by a fungal pathogen, *Pyricularia grisea* (Ma, 2006; Lemus and Tomaso-Peterson, 2010; Rahman *et al*., 2015). Disease development is highly depended on climatic conditions and cropping management practices, such as nitrogen inputs, proper mowing, irrigation and cultivar susceptibility (Williams *et al.*, 2001; Uddin *et al.*, 2004; Douhan *et al.*, 2011). The most effective way to control this pathogen has been the use of fungicides (Ma, 2006). However, dependence on chemical application has resulted in pathogen resistance to fungicides within the pathogen population (Vincelli and Dixon. 2002; Kim *et al*., 2003; Li, 2013).

Application of biological control agents (BCAs) is an important strategy in crop protection against plant pathogens. It is a desirable practice in line with the principles of sustainable agriculture. The use of BCAs as an alternate or supplement control tool may reduce the risk of fungicide resistant strains developing in the fungal populations (Hu *et al*., 2014). Bacteria belonging to the genus *Bacillus* are ideal candidates as BCAs to be used in farming systems (Ji *et al*., 2006). In particular, some strains are able to synthesis various antimicrobial compounds against fungi, and enhance the growth and defense responses in host plants. Additionally, they form endospores that allow them to resist adverse environmental conditions as well as allowing for easy formulation and storage as commercial products. In some studies *Bacillus* isolates have demonstrated the ability to suppress the following plant diseases under field conditions: *Cercospora* leaf spot on sugar beet (*Beta vulgaris* L.) (Collins and Jacobsen, 2003); *Verticillium* wilt of potato (Uppal et al., 2008); necrosis virus disease of sunflower (Srinivansan and Mathivanan, 2009); *Colletotrichum acutatum* of postbloom fruit of citrus (Kupper et al., 2012); and *Sclerotinia sclerotiorum* on oilseed rape (*Brassica napus* L.) (Hu et al., 2014).

Silicon (Si) fertilization appears to enhance disease resistance in plants. It can contribute to the reduction in intensity of various economically important diseases in monocotyledon and dicotyledon plant species which include: tomato (*Lycopersicon esculentum* Mill) (Kiirika *et al*., 2013), cucumber (*Cucumis sativus* L.) (Ferreira *et al*.,

2015), wheat (*Triticum aestivum* L.) (Silva *et al*., 2010), sorghum (*Sorghum bicolor* L.) (Resende *et al*., 2009) and rice (*Oryzae sativa* L) (Rodrigues and Datnoff, 2005). Plant resistance to disease is due either to an accumulation of absorbed Si in the epidermal tissue forming a mechanical barrier restricting fungal penetration or expression of pathogenesis–induced host defense responses (Kurabachew and Wydra, 2014).

Many integrated diseases management strategies aim at reducing reliance on fungicides and rely more on less toxic products that have different modes of action. Integrated control programs that include the application of antagonistic microorganism provide a promising strategy for the management of plant diseases. Several studies have shown the effectiveness of a large number of plant beneficial microorganisms used in combination with defense response inducing chemicals including methyl jasmonate (Yao and Tian, 2005); sodium bicarbonate (Yao *et al*., 2004), salicylic acid (Farahani and Etebarian, 2011) and silicon (Tian *et a*l., 2005; Farahani *et a*l., 2012).

Therefore, the aim of this chapter was to investigate whether *Trichoderma harzianum* Strain B77 (T.77), *Bacillus cereus* Strain I48 and *Bacillus* sp Strain M1, applied alone or combined with liquid potassium silicate KSil could reduce GLS severity under field conditions.

5.2. Materials and Methods

5.2.1. Potassium Silicate (KSil)

Potassium silicate (KSil) was applied in a form of a liquid formulation (K_2SiO_3), Agrisil K50 (PQ Corporation, South Africa) containing 33 g kg^{-1} potassium as K and 96 g kg^{-1} silicon.

5.2.2. Source of seed

The perennial ryegrass seed *Lolium perenne* (cv Arrow) was provided by Barenbrug Seeds (Pty) Ltd, Cape Town, South Africa. The annual ryegrass seed *Lolium multiflorum* (Barrextra) was supplied by Farmers Agri-Care (Pty) Ltd, Howick, South Africa

5.2.3 Inoculum production for field trials

a) Pathogen inoculation

An isolate of *P. grisea* was grown on oat meal agar (OMA) for 7-14 d. Agar blocks carrying actively growing mycelia were transferred to OMA plates and incubated in the dark at 28°C for 7-14 d. Using a scalpel, the mycelia were removed by scraping the surface of the culture plates. The fungal plates were then placed under continuous white light at 20°C for 7-14 d in order to induce sporulation. The conidia were harvested and suspended in sterile distilled water. Using a haemocytometer, a final concentration of conidia was adjusted to approximately $1x10^5$ conidia ml^{-1}.

b) Bacillus Isolate

The bacterial isolates, *Bacillus cereus* I48 and *Bacillus* sp. M1, showed potential in reducing GLS severity under greenhouse conditions and as a result were selected for further tests in the field (Chapter Three). Fresh cultures were prepared from frozen stock cultures by subculturing the individual isolates on Nutrient Agar (NA) plates and incubated at 28°C for 24 h. The bacterial cultures were resuspended in a 1.5% solution of Break-Thru® (Universal Crop Protection (Pty) Ltd. Kempton Park, South Africa) in tap water. Cell density was determined using a Helber Bacterial Counting Chamber (Paul Marienfield-Superior GmbH & Co KG, Germany) and the concentration of 10^8 cells ml^{-1} was established.

c) Trichoderma harzianum Strain B77

Trichoderma harzianum B77 (T.77) is a commercially formulated biocontrol agent that is manufactured by Plant Health Products (Pty) Ltd, Nottingham Road, South Africa. The T.77 treatment was prepared by suspending 6g of the product in six liters of tap water, as recommended by the manufacturer, making up a suspension containing $1x10^9$ conidia ml^{-1}.

5.2.4. Efficacy of biological control agents and potassium silicate

The field experiments were conducted at Ukulinga Research Station, University of KwaZulu-Natal, Pietermaritzburg, South Africa (29°24’ E, 30°24’ S, altitude 845 m a.s.l.). The field site was pre-treated with Basagran® (3 L ha^{-1}) to remove broadleafed weeds and Karate EC (3 L ha^{-1}) to remove cutworms, followed by manually removing all

vegetation. Ryegrass seeds were planted in a broadcast manner at a rate of 25 kg ha^{-1}. The field trials were carried out by applying alone or in combination with KSil the following biocontrol agents: T.77, *B. cereus* I48 on perennial ryegrass and *Bacillus* spp M1 on annual ryegrass. The biocontrol suspensions were adjusted to a final concentration of approximately $1x10^9$ conidia ml^{-1} for T.77 and $1x10^8$ cells ml^{-1} for the *Bacillus* strains. The pathogen inoculum was applied 2d after treatment with the biocontrol agents. Treatments were applied by spraying biocontrol suspensions onto plant foliage using a Ryobi pressure sprayer GS-600 (0.15 – 0.3 Mpa) (Stevens & Co Pty (Ltd)) on four-week old ryegrass plants. KSil was applied at the following concentrations: 200 ppm on annual ryegrass and 300 ppm on perennial ryegrass. The plants were drenched separately with each KSil concentration one week after germination and then weekly thereafter. The fungicide azoxystrobin was applied at a rate of 600 ml ha^{-1} in tap water. The fungicide was applied as soon as symptoms appeared. The experiments were arranged in a complete randomised blocks design with three replicates (plots) per treatment. Each plot had an area of 2.6 m^2 (2.3 m x 1.14 m). In order to reduce errors due to any plot-border effects, the area assessed was in the center (1.15 m x 0.7 m) of each plot. Disease severity ratings were made 7d after pathogen inoculation and every week thereafter for 6wk.

5.2.5. Disease assessment

Gray leaf spot severity was assessed visually on a scale of 0 to 10, where 0 = no disease; 1= 1-10% of whole plant area necrotic and blighted; 2= 11-20%; 3= 21-30%; 4= 31-40%; 5= 41-50%; 6= 51-60%; 7= 61-70%; 8= 71- 80%; 9= 81-90%; 10= 91-100%. The infected plant area was measured weekly for six weeks. The mid-point values were used to calculate the Area Under the Disease Progress Curve (AUDPC) for all treatments (Shaner and Finney, 1977) before statistical analysis.

5.2.6. Data analysis

The AUDPC values (LN transformed) and the final disease severity values (arcsine transformed) were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc., 2011). Treatment means were separated using Duncan Multiple Range Test (DMRT) at the 5% probability level (P = 0.05).

5.3. Results

5.3.1. Efficacy of biological control agents, potassium silicate and their combined application on annual ryegrass

The combination treatments did not significantly reduce GLS severity when compared to the pathogen inoculated Control (Table 5.1). *Bacillus* spp (M1) performed reasonably well in reducing GLS severity. It caused the greatest reduction in GLS severity of 22.0% of the combination treatments, or when KSil and T.77 were applied alone. No significant differences were found between the treatments and the pathogen inoculated control. The fungicide treatment caused the greatest reduction in GLS severity, significantly reducing disease severity by 44% (P = 0.002). Overall, azoxystrobin out performed KSil and the biocontrol agents, applied alone and in combination, in reducing GLS severity in the field. There were no significant differences in AUDPC values between treatments (Table 5.1).

5.3.2. Efficacy of biological control agents, potassium silicate and their combined application on perennial ryegrass

Bacillus cereus I48 applied alone was found to significantly reduce GLS severity (P = 0.03). KSil applied in combination with *B. cereus* did not reduce GLS severity. KSil and T.77 when applied alone and in combination with KSil were not successful in controlling GLS, reducing GLS severity by only 10.7%. No significant differences were found between these treatments and the pathogen inoculated control. Azoxystrobin significantly reduced GLS severity by 53.6% (P = 0.0002). The AUPDC value of azoxystrobin was significantly lower than the pathogen inoculated control (P = 0.0058). Overall, azoxystrobin and *B. cereus* I48 applied alone performed better in reducing GLS severity than any of the combination treatments or KSil and T.77 applied alone.

Table 5.1. Efficacy of *Bacillus* sp. M1, *T. harzianum* strain B77 (T.77), potassium silicate (KSil) and the combined application of KSil with biocontrol agents on gray leaf spot on annual ryegrass in the field.

	Experiment I		
Treatment	**Percent Foliar disease severity[1,2,3]**	**% reduction of GLS**	**AUDPC[3,4]**
Pyricularia grisea	45.5a	0	857.5a
Azoxystrobin	25.5b	44.0	647.5a
KSil	42.2a	7.3	927.5a
T.77	38.8a	14.7	845.5a
Bacillus sp. (M1)	35.5ab	22.0	600.8a
KSil + T.77	38.8a	14.7	752.5a
KSil + *Bacillus* sp. (M1)	42.2a	7.3	915.8a
F-ratio	2.76		0.82
P-value	0.05		0.58
%CV	10.7		5.3

[1]Visual ratings of foliar disease severity (0 – 100). Numbers are arcsine transformed.
[2]The first visual ratings were made on whole plant at 1 week after inoculation with *Pyricularia grisea.* Ratings were made weekly thereafter for 6 weeks [1 = 0%; 2 = 1 – 10%; 3 = 11 – 20%; 4 = 21- 30%; 5 = 31 – 40%; 5 = 41- 50%; 6 = 51- 60%; 7 = 61 – 70%; 8 = 71 – 80%; 9 = 81 – 90%; 10 = 91 – 100%]
[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)
[4]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates. Numbers are LN transformed.

Table 5.2. Efficacy of *Bacillus cereus* I48, *T. harzianum* strain B77 (T.77), potassium silicate (KSil) and the combined application of KSil with biocontrol agents on gray leaf spot on perennial ryegrass in the field.

Treatment	Experiment I		
	Percent Foliar disease severity[1,2,3]	**% reduction of GLS**	**AUDPC[3,4]**
Pyricularia grisea	62.2a	0	1417.5a
Azoxystrobin	28.8c	53.6	554.2b
KSil	55.5ab	10.7	1219.2a
T.77	55.5ab	10.7	1184.2a
Bacillus cereus (I48)	45.5b	36.6	1125.8a
KSil + T.77	55.5ab	10.7	1347.5a
KSil + *Bacillus cereus* (I48)	62.2a	0	1207.5a
F-ratio	5.89		2.77
P-value	0.003		0.04
%CV	10.6		4.3

[1]Visual ratings of foliar disease severity (0 – 100). Numbers are arcsine transformed.
[2] The first visual ratings made on whole plant at 1 week after inoculation with *Pyricularia grisea.* Ratings were made weekly thereafter for 6 weeks. [1 = 0%; 2 = 1 – 10%; 3 = 11 – 20%; 4 = 21- 30%; 5 = 31 – 40%; 5 = 41- 50%; 6 = 51-60%; 7 = 61 – 70%; 8 = 71 – 80%; 9 = 81 – 90%; 10 = 91 – 100%]
[3]Within each column, values followed by the same letter indicate no significant difference at P =0.05, according to Duncan Multiple range test (DMRT)
[4]AUDPC = Area Under the Disease Progress Curve based on disease severity on six assessment dates. Numbers are LN transformed.

5.4. Discussion

Although KSil provided promising results in reducing GLS severity under greenhouse conditions (Chapter 4) when applied alone KSil was ineffective in the field. The possible reason why KSil was ineffective in the field was that pathogen infection may have taken place before KSil could prime for sufficient host resistance against the pathogen. Brecht *et al*. (2007) found that silicon affected only one component of resistance, namely lesion number of GLS on St. Augustine grass. This led to the assumption that silicon has no influence on the rate of colonization, symptom development or conidial production. Further to that, silicon is more effective in the range of 20 – 25°C. Its effectiveness reduces with increasing temperatures (Scheurger and Hammer, 2003). High temperatures experienced in the field may have reduced the efficacy of silicon against GLS severity on ryegrass.

Trichoderma harzianum strain B77 (T.77) was ineffective in controlling GLS on annual and perennial ryegrass in the field. The same was true under greenhouse conditions (Chapter 3). This suggests that T.77 does not have the potential to compete in the phylloplane of ryegrass. Additionally, environmental factors such as wet-dry cycling conditions, high-low temperatures, variations in humidity and U.V. radiation have been identified to have a negative effect on the efficacy of formulated biological control products (Wiwattanapatapee *et al*., 2013).

Our research showed that biocontrol agents combined with KSil were ineffective in controlling GLS disease on ryegrass under field conditions. To the best of our knowledge there are no studies on the effects of silicon combined with biocontrol agents on GLS severity of ryegrass. However, certain studies have shown that silicon in combination with yeast antagonists provided a synergistic effect and that the integrated treatments were effective in controlling various plant pathogens. De Curtis *et al*. (2012) demonstrated that combined field application of yeast biocontrol agents and silicon had the potential to control wheat powdery mildew. Also silicon fertilization combined with yeast has been successful in controlling postharvest diseases on sweet cherry fruit (Qin and Tian, 2005) and on apples (Farahani and Etebarial, 2012; Farahani et al., 2012). Given the success of the yeast antagonists combined with silicon, perhaps future

studies should aim at investigating the efficacy of yeast biocontrol agents combined with silicon against GLS of ryegrass.

Among the bacterial antagonists tested, *Bacillus* spp M1 was the best in reducing GLS severity in the field on annual ryegrass. Even though *B. cereus* did provide some control against GLS in the field, the results were not significantly different from the pathogen inoculated control. Some biological control agents are able to control diseases effectively and are environmentally safe. However, their performance is influenced by environmental conditions such as solar radiation, temperature and pollution, as well as the presence of other microorganisms, and are generally less predictable than chemical control.

When compared to the bacterial antagonists, azoxystrobin provided consistent protection against GLS severity in the field. This may be due to the immediate action of the fungicide, which lasts 21-35 days. Azoxystrobin is among the most effective fungicides for GLS. It has a single-site mode of action on the pathogen through the inhibition of the fungal mitochondrial respiration (Ma, 2006). Despite its success, a strong reliance on this chemical may result in failure to provide adequate disease protection due to resistance (Ma, 2006).

In summary, azoxystrobin significantly reduced GLS severity on both annual ryegrass and perennial ryegrass. *Bacillus cereus* I48 significantly reduced GLS severity on perennial ryegrass. Although Bacillus sp. M1 reduced GLS severity on annual ryegrass, the reduction was not significant. KSil applied alone or in combination with either of the biological control agents was ineffective in the field. T. *harzianum* strain B77 (T.77) was ineffective at controlling the disease in both greenhouse and field experiments.

Integration of biological control agents with chemical control has been successful in controlling several plant diseases (Brannen and Kenney, 1997; Korsten *et al*., 1997; Kondoh *et al*., 2001; Cook et al., 2002; Jacobsen *et al*., 2004; Anand *et al*., 2010; Zeng *et al*., 2012, Peng *et al*., 2014). Perhaps combining the bacterial antagonists with

azoxystrobin should be the aim of future research. This might reduce the levels of fungicide application, or frequency.

5.5. References

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Thesis Overview of the Major Research Findings and their Implications

Introduction

Gray leaf spot (GLS) is a destructive disease on annual (*Lolium multiflorum* L.) and perennial (*Lolium perenne* L.) ryegrass caused by *Pyricularia grisea*. The best disease management practices for controlling GLS include chemical and cultural control. However, under rapid disease development, cultural management practices do not provide adequate control of GLS. The development of resistance to fungicides by fungal pathogens limits the effectiveness of chemicals in controlling the disease. In addition, there is a growing concern regarding the dependence of farmers on chemical control which results in environmental pollution. It has therefore become important to find alternative control measure for the management of GLS of ryegrass.

The aim of this study was to use biological control agents and potassium silicate (KSil) to manage GLS on ryegrass. The specific objectives were as follows: 1) To isolate, screen *in vitro* and identify potential bacterial biological control agents against *P. grisea*; 2) To evaluate the effects of selected bacterial biological control agents against GLS caused by *P. grisea* in the greenhouse; 3) To evaluate four different concentrations and two application frequencies of KSil to suppress GLS disease under greenhouse conditions; 4) To evaluate the optimum concentration and application frequency of KSil in combination with biological control agents to suppress GLS of ryegrass under field conditions

Chapter 2: Isolation and *in vitro* screening of potential biological control agents against gray leaf spot of ryegrass

Major findings:

- Eighty-seven bacteria biocontrol agents were isolated from the phyllosphere of various graminaceous hosts and screened *in vitro* for their antagonistic ability against *P. grisea*.
- Nine isolates were identified as the most promising antagonists against *P. grisea in vitro*.
- The bacterial strains were identified using the 16S rRNA sequence analysis and four of the isolates were identified as *Bacillus* spp, two as *Pseudomonas* spp, one as *Bacillus amyloliquefaciens* and one as *Bacillus cereus*.
- The commercial strains of *Trichoderma harzianum* Strain T.kd (T.kd) and *Trichoderma harzianum* Strain B77 (T.77) inhibited mycelial growth of *P. grisea in vitro*.
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Implications:

Of the 87 bacterial isolates screened 9 isolates showed potential as biocontrol agents. This suggests the importance of subjecting the isolates to a rigorous screening process in order to select the most promising biocontrol agents.

The dual test results suggests that Bacillus spp, *Pseudomonas* spp and the commercial strains of *T. harzianum* are good biocontrol candidates for further studies because they resulted in the greatest suppression of the pathogen in dual-plate assays.

Chapter 3: *In vivo* screening of biological control agents against gray leaf spot of ryegrass under greenhouse conditions

Major findings:

- There were inconsistencies in the performance of the biocontrol agents tested on annual ryegrass in the greenhouses. In Experiment 1 *Bacillus* spp S6 and B57

reduced GLS severity in the greenhouse by 26 and 34%, respectively. In Experiment 2 *Bacillus* spp M1 reduced GLS severity by 20% while *B. amyloliquefaciens* B7 and *Pseudomonas* spp I74 both reduced GLS severity by 19% in the greenhouse. However, no significant differences were found between the bacterial treatments and the pathogen inoculated control.

- On perennial ryegrass, *B. cereus* I48 and *Bacillus* spp S6 consistently reduced GLS severity in both experiments under greenhouse conditions, *B. cereus* I48 reduced severity by 35% in Experiment 1, and by 41% in Experiment 2. *Bacillus* spp S6 reduced disease severity by 30% in Experiment 1 and by 39% in Experiment 2. No significant differences were found between the bacterial treatments and the pathogen inoculated control. The level of control provided by the biocontrol agents was comparable to that of azoxystrobin.
- *T. harzianum* Strains T.kd and T.77 were not effective in reducing GLS severity on perennial ryegrass and annual ryegrass under greenhouse conditions.
- Azoxystrobin reduced GLS severity in annual ryegrass by 28% in Experiment 1 and by 32% in Experiment 2. In perennial ryegrass, azoxystrobin reduced GLS by 35% in Experiment 1 and by 54% in Experiment.

Implications:

Some of the bacterial antagonists selected for their strong inhibitory ability against *P. grisea in vitro* performed poorly when tested on ryegrass under greenhouse conditions. Conversely, some strains that performed poorly *in vitro* performed better than expected under greenhouse conditions. It is important to subject potential biocontrol agents to screening in the greenhouse in order to select the best candidate for field studies.

The bacterial biocontrol agents, particularly on annual ryegrass, were inconsistent in their performance during the greenhouse studies. The reason could be that the isolates were obtained from a non-host leaf surface, suggesting that the leaf surface of the ryegrass may not have been favourable for the establishment and proliferation of the bacterial biocontrol agents.

The *T*. *harzianum* strains were ineffective in reducing GLS of ryegrass under greenhouse conditions, suggesting that they are unable to successfully colonise and multiply on the leaf surface.

Bacillus spp M1 and *B. cereus* I48 that were able to reduce GLS on ryegrass consistently, and were comparable to of the control provided by azoxystrobin. This suggests that the bacterial biocontrol agents show great potential to be used in the field.

Chapter 4: Efficacy of different concentrations and frequency application of potassium silicate against gray leaf spot of ryegrass under greenhouse conditions

Major findings

- Three potassium silicate concentrations significantly ($P < 0.05$) reduced GLS severity under greenhouse conditions in both annual and perennial ryegrass. Concentrations of 100 ppm:1 (applied once a week), 200 ppm:2 (applied twice a week) and 300 ppm:1 (applied once a week) reduced GLS severity by 17.0, 19.3 and 23.5%, respectively, in perennial ryegrass while concentrations of 100 ppm:2 (applied twice weekly), 300 ppm:1 (applied once a week) both reduced GLS severity by 27.3%; and 200 ppm:1 (applied once a week) reduced GLS severity by 29.8% on annual ryegrass.

Implications:

A reduction in GLS severity on ryegrass as a result of KSil suggests that it has the potential to be used in the field to reduce GLS severity. KSil is able to act as a physical barrier that hinders pathogen entry through the epidermis or functions as a signal that accelerates the induction of disease resistance in plants triggered by the pathogen. Therefore, KSil can be used as part of an integrated disease management strategy.

Chapter 5: Integrated management of gray leaf spot of ryegrass with biological control agents and potassium silicate under field conditions

Major findings:

- None of the combined treatments resulted in a reduction of GLS severity in the field.
- KSil applied alone did not effectively control GLS on ryegrass in the field.
- *T. harzianum* Strain T.77 did not result in a reduction in GLS severity on both annual and perennial ryegrass in the field.
- Application of *B. cereus* (I48) on perennial ryegrass and *Bacillus* spp (M1) on annual ryegrass resulted in a reduction in GLS severity in the field.
- Azoxystrobin caused a significant reduction in GLS severity on annual ryegrass and perennial ryegrass in the field.

Implications:

The combination treatments were relatively ineffective in reducing GLS severity. This suggests that there might be little antagonistic effect in controlling GLS when the two treatments are combined.

Although KSil was able to reduce GLS severity under greenhouse conditions, it was unsuccessful in the field. Environment factors could have affected the efficacy of KSil against GLS in the field. Also the disease pressure may have been too high and this could have delayed priming of disease resistance in plants by KSil.

T. harzianum Strain T.77 was not able to reduce GLS on ryegrass in either greenhouse or field trials, suggesting that T.77 was probably unable to effectively colonise and multiply on the leaf surfaces of ryegrass.

Bacillus spp were effective in reducing GLS severity in the field. These biocontrol agents should be screened again in the field in order to confirm their effectiveness. The bacterial antagonists showed potential as biocontrol agents that could possibly be used as part of an integrated disease management strategy. Azoxystrobin was able to significantly reduce GLS severity in the field as expected. However, its continuous use would pose a risk of fungicide resistant strains emerging within the *P. grisea* population.